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Game Changer: a Mechanical Insecticide for Mosquitoes, Sand Flies, Filth Flies and Other Arthropods from Volcanic Rock

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14. ABSTRACT
Mosquitoes, filth flies, sand flies and other arthropods transmit to the deployed warfighter microbial pathogens that cause illness and death. One of the most important approaches to prevent disease is the control of the pest arthropods. The most common method has been the development and use of chemical pesticides. Because of natural selection, the use of pesticides result in pest populations that become resistant. As a result, new control methods must be in constant development. Furthermore, non-chemical natural approaches are often more acceptable to users. We discovered the use of a non-chemical method for the control of mosquitoes, flies, and ticks using mechanical insecticides, i.e., specific bio-minerals made by diatoms and a bio-mimic made from volcanic glass. In this proposal, we showed proof of concept for the use of this technology as a topical killing agent against the target pests under study, conducted research to support the registration of these new products, and submitted registration packages to the US EPA and WHO (some granted, others pending). The products are being commercialized under the names Celite 610 and Imergard, respectively. The research effort unexpectedly resulted in other discoveries including a new insect and tick repellent (EPA registered April 2023), human diseases unknowingly being transmitted by flies and mosquitoes, and the discovery of a new human disease in the US.

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Introduction

Mosquitoes are the foremost disease vectors and nuisance pests for U.S. military personnel and present the most obvious insect threat (Ciminera et al., 2007; Kitchen et al., 2009). They transmit three of the most serious vector-borne diseases that jeopardize U.S. forces, i.e., malaria, dengue, and viral encephalitis. **Phlebotomine sand flies** transmit protozoan parasites (*Leishmania* spp.) as well as bacterial (*Bartonella bacilliformis*) and viral pathogens. Human leishmaniases are the most significant vector-borne illnesses on a global basis following malaria and dengue (Ready, 2013). **Diarrheal diseases** can quickly become a significant public health concern in temporary living conditions, including military camps (Prendergast et al., 2002). Such diseases may significantly impact mission readiness with 10% or more of service personnel affected at a given time. Proper sanitation (e.g., latrine position) and personal hygiene (e.g., hand-washing) can be implemented easily, but enteric pathogens are still readily transmitted by filth flies. Filth flies are flies that develop and feed as adults on feces, carrion and foodstuffs and become mechanical vectors of enteric disease, causing a devastating impact on the readiness of combat troops. The control of these pests is essential to preserve soldier health.

The most common method for mosquito and fly control has been the development and use of chemical pesticides. However, the use of pesticides results in the selection of pest populations that become resistant to the pesticide. As a result, new control methods must be in constant development. Furthermore, non-chemical natural approaches are often more acceptable to users. We discovered the use of a non-chemical method for the control of mosquitoes, flies, and ticks using mechanical insecticides. These minerals when transferred to the surface of the insect causes rapid water loss by disrupting the natural water protection layer of the cuticle. It is hypothesized this occurs by abrasion of the cuticle and/or absorption of the lipid water proofing, outer layer of the cuticle. Our research is concentrated on two mineral types, a bio-mineral, diatomaceous earth (DE), made by specific species of diatoms (mined from deposits in the earth) and a bio-mimic made from volcanic glass. Our research was to show proof of concept for the use of this technology as a topical killing agent against the target pests under study, the conduct of research to support the registration of these new products, and the submission of registration packages to the US EPA and WHO. The products are being commercialized under the names Celite 610 (DE) and Imergard (volcanic glass), respectively. The research effort unexpectedly resulted in other discoveries including a new insect and tick repellent (EPA registered April 2023); a non-insecticidal bed net for mosquito control; new fumigant; new pesticide for head lice control; potential diseases unknowingly transmitted by flies and mosquitoes; and the discovery of a new human disease in the US, scrub typhus. These accomplishments will be summarized in the body of this final report by "category of work" and most have been published and/or patented (referenced in this report and with pdfs of the full documents in the appendix that follows). A bibliography of all of the work published is also provided in this report along with outcomes, conclusions, presentations, patents, licenses and Ph.D. dissertations completed.

Body of Report

1. Mosquito findings, Imergard

Deguenon, J. M., C. Riegel, E. R. Cloherty-Duvernay, K. Chen, D. A. Stewart, B. Wang, D. Gittins, L. Tihomirov, C. S. Apperson, M. G. McCord and R. M. Roe. 2021. New mosquitocide derived from volcanic rock. J. Med. Ent. 58, 458–464.

During laboratory screening of industrial minerals for insecticide activity against *Anopheles gambiae,* we discovered a novel mechanical insecticide derived from volcanic rock (MIVR) with potential use as a residual spray. In modified WHO cone tests (partial Phase I studies), the time to 50% mortality was 5 h under high-humidity conditions. MIVR treated surfaces demonstrated no mosquito repellency. In field studies where the mechanical insecticide was applied to wood using standard spray equipment and then placed under stilt homes in New Orleans, LA, the residual activity was >80% after 9 wk against *Aedes aegypti* (L.), *Aedes albopictus* and *Culex quinquefasciatus* (Say) (Diptera: Culicidae) (with similar efficacy to a positive chemical insecticide control). In scanning electron microcopy studies, the MIVR was transferred as particles mostly to the legs of the mosquito. This wettable powder made from volcanic rock is a mechanical insecticide representing a potential new mode of action different from current chemistry for mosquito control and is in commercial development under the trade name Imergard™WP as an indoor and outdoor residual spray.

Deguenon, J. M., R. Azondekon, F. R. Agossa, G. G. Padonou, R. Anagonou, J. Ahoga, B. N'dombidje, B. Akinro, D. A. Stewart, B. Wang, D. Gittins, L. Tiomirov, C. S. Apperson, M. G. McCord, M. C. Akogbeto and R. M. Roe. 2020. Imergard^{TM}WP: a non-chemical alternative for an indoor residual spray, effective against pyrethroid-resistant *Anopheles gambiae* (s.l.) in Africa. Insects. 11,322; doi: 10.3390/insects11050322.

The efficacy of a novel mechanical insecticidal mineral derived from volcanic rock, Imergard^{TM}WP, was investigated to determine its efficacy as a stand-alone residual wall spray and as a mixture with deltamethrin (K-Othrine® Polyzone) in experimental huts in Cove, Benin (a WHO Phase II study). The evaluation was conducted with susceptible (Kisumu) and wild-type *Anopheles gambiae* (s.l.). Deltamethrin applied alone demonstrated 40–45% mortality (at 72 h post-exposure) during the first four months, which declined to 25% at six months for wild *An. gambiae* from Cove. Imergard^{TM}WP alone and mixed with deltamethrin, under the same assay conditions, produced 79–82% and 73–81% mortality, respectively, during the same six-month period. Imergard^{TM}WP met the 80% WHO bio-efficacy threshold for residual activity for the first five months with 78% residual activity at six months. Imergard^{TM}WP can be used as a mixture with chemical insecticides or as a stand-alone pesticide for mosquito control in Africa.

Current status. Phase III community studies were conducted in Africa by the company Imerys where 4000 homes were treated. Mosquito control was achieved by WHO standards for 11 months. Imerys also conducted additional Phase I work to support the WHO submission with

an independent contractor. The WHO registration package was reviewed, we responded to questions about our Phase II studies, and the WHO decision for approval is pending.

2. Filth fly control by Imergard and Celite.

Chen, K., J. M. Deguenon, G. Cave, S. S. Denning, M. H. Reiskind, D. W. Watson, D. A. Stewart, D. Gittins, Y. Zheng, X. Liu, C. S. Mouhamadou and R. M. Roe. 2021. New thinking for filth fly control: residual, non-chemical wall spray from volcanic glass. Med. Vet. Entomol. doi: 10.1111/mve.12521.

The mechanical insecticide made from volcanic glass and originally developed to control mosquitoes (Imergard™ WP; ImG) was investigated for control of adult grey flesh flies, *Sarcophaga bullata* (Parker), secondary screwworms, *Cochliomyia macellaria* (F.), and house flies, *Musca domestica* L. In a modified WHO cone test device, the time to 50% mortality (LT50) when applied at 5 g/m^2 (tested at 30 ◦C and 50% relative humidity (rH)) was 7.1, 4.3 and 3.2 h, respectively. When knockdown was included, the LT50s were 5.5, 1.5 and 2.8 h, respectively. Application rates of 1.25 and greater g/m^2 had the shortest LT50s. The time to the LT50 increased for *M. domestica* as rH increased, but ImG was still active at the highest rH tested of 70%. Scanning electron micrographs showed ImG was present on all body parts, unlike that for mosquitoes where it was found mostly on the lower legs. These studies on the use of Imergard WP against flies suggest this could be an alternative method for filth fly control.

Cave, G. L., K. Chen, S. S. Denning, D. W. Watson, and R. M. Roe. Submitted. Comparative efficacy and mode of action of the bio-mineral produced by diatoms, Celite 610, against three species of filth flies. Insects 2023, 14, x. https://doi.org/10.3390/xxxxx.

A modified WHO cone test was used to determine the efficacy of the minerals produced by diatoms found in the commercial product, Celite 610, against house fly (*Musca domestica*), secondary screwworm (*Cochliomyia macellaria*), and grey flesh fly (*Sarcophaga bullata*) adults. Mortality was observed as early as 2 h with 50% and 90% mortality observed in as little as 3.5 and 4.6 h (the LT50 and LT90), respectively, in house flies. The LT50 and LT90 increased as the size of the fly species increased (*M. domestica* to *C. Macellaria* to *S. bullata*) and also increased for all three species when the relative humidity increased from 50 to 70% at 30˚C. These results suggest dehydration was the mode of action; however, scanning electron micrographs of live secondary screwworm adults 2 h after Celite exposure, showed the flies were evenly covered with the mineral with no obvious damage to the cuticle. Proof of concept was demonstrated that these minerals can be used for filth fly control.

Current status. COVID restrictions prevented the conduct of field studies. Small cage studies (data not published) further showed proof of concept that a small treated surface was highly effective in filth fly control, even when the flies were provided a water and food source. Our data also showed the mineral was effective against filth flies by misting. An EPA registration was granted for the use of Celite as a residual spray treatment for flies. An EPA registration for Imergard is currently on hold, waiting on the WHO decision for mosquitoes. WHO and EPA had no concerns about the safety of Imergard.

3. Sandfly control by Imergard and Celite.

Chen, K., J. M. Deguenon, R. D. Lawrie and R. M. Roe. 2023. Biomolecular minerals and volcanic glass bio-mimics to control adult sand flies, the vector of human Leishmania protozoan parasites. Biomolecules, 13, 1235. https://doi.org/10.3390/biom13081235.

The biomineral produced by diatoms (diatomaceous earth, DE; Celite) and a volcanic glass bio-mimic (Imergard) have been shown by our group to be efficacious against mosquitoes and filth flies but never studied for the control of sand flies. In a modified WHO cone test, 50% of adult *Phlebotomus papatasi* sand flies at 29 ± 1 °C, 55 ± 5% RH and 12:12 LD, when exposed to Imergard and Celite, were dead in 13.08 and 7.57 h, respectively. Proof of concept was established for the use of these biominerals for sand fly and leishmaniasis disease control. Using a light source as an attractant to the minerals had no significant effect on the LT50, the time to 50% mortality. The LT50 at a higher relative humidity of 70 ± 5% increased to 20.91 and 20.56 h for Imergard and Celite, respectively, suggesting their mode of action was dehydration. Scanning electron microscopy of dead sand flies showed high coating levels of Celite only on the sides of the thorax and on the tarsi, suggesting an alternative mode of action for mechanical insecticides.

Current status. COVID restrictions prevented the conduct of field studies at NC State University. Military personnel were sent back to the US from the US Army, USAMRD-A, in Kenya, Africa during COVID and the large cage studies were not conducted. Our cone tests established Celite and Imergard was effective against sand flies, but the activity was less than our filth fly and mosquito studies. Our hypothsis is that sand flies are adapted to low humidity environments and exposure to small sand particles with long body setae. More research is needed as described for the studies in Kenya in our proposal to understand how to apply the technology for sand fly control in the field. An EPA registration was granted for the use of Celite as a residual spray treatment for flies, which includes sand flies. Research is continuing on proof of concept in using Celite and Imergard in traps for mosquitoes, which we hypothesize, will be translational to filth flies and sand flies.

4. Other arthropod (tick) control by Imergard and Celite.

Richardson, E.A.; L. Ponnusamy and R. M. Roe. 2022. Mechanical acaricides active against the blacklegged tick, *Ixodes scapularis*. Insects 2022, 13, 672. https:// doi.org/10.3390/insects13080672. 11pp.

Ixodes scapularis, also known as the blacklegged tick or deer tick, is the vector of the bacteria that causes Lyme disease in humans, the most common vector-borne disease in the US. Synthetic chemical pesticides are used to control ticks. Environmentally friendly, new methods are needed to manage chemical pesticide resistance. Although the above studies demonstrate the use of Imergard and Celite for Diptera (mosquitoes and flies), the use of mechanical insecticides have never been examined for use against ticks. We evaluated the efficacy of the industrial mineral, Celite 610, an amorphous silica, against unfed *I. scapularis* nymphs. Celite is found in nature and has a mechanical, non-toxic mode of action. Dipping ticks into Celite for 1–2 s resulted in 90% mortality in as little as 69 min. Scanning electron microscopy suggested that one mode of action could be the physical obstruction of respiration. We developed another industrial mineral made from volcanic glass, Imergard™ WP, for mosquito and fly control. In studies here, Imergard had similar activity as Celite against the deer tick. This research, although needing further study, suggests that industrial minerals could be a new, safe ("found in toothpaste") and persistent ("it's rock") alternative to chemical pesticides to control ticks.

Cave, G. L., E. A. Richardson, K. Chen, D. W. Watson and R. M. Roe. 2023. Acaricidal biominerals and mode-of-action studies against adult blacklegged ticks, *Ixodes scapularis*. Microorganisms, 11, 1906. https://10.3390/microorganisms11081906.

Volcanic glass, Imergard, was developed to mimic the insecticide mode of action of the minerals from diatoms (diatomaceous earth, DE) for the control of malaria mosquitoes in Africa. However, studies on the use of these minerals for tick control are minimal. In a dipping assay, which was put into DE (Celite), the times of 50 and 90% death of adult female *I. scapularis* were 7.3 and 10.5 h, respectively. Our mimic of DE, Imergard, killed ticks in 6.7 and 11.2 h, respectively. In a choice-mortality assay, ticks moved onto a treated surface of Imergard and died at 11.2 and 15.8 h, respectively. Ticks had greater locomotor activity before death when treated by dipping for both Imergard and Celite versus the no-mineral control. The ticks after making contact with Imergard had the mineral covering most of their body surface shown by scanning electron microscopy with evidence of Imergard inside their respiratory system. Although the assumed mode of action of Imergard and Celite is dehydration, the minerals are not hygroscopic, there was no evidence of cuticle damage, and death occurred in as little as 2 h, suggesting minimal abrasive action of the cuticle.

Current status. Semi-field and field studies are needed in the future to examine the practical use of Imergard and Celite for tick control, and studies needed to examine their effect on tick breathing and respiratory retention of water. Also studies are needed to examine the use of these minerals in nesting materials to control ticks on rodents, especially relative to the control of Lyme disease. This work is in progress as part of new DWFP funding.

5. Research progress made from earlier DWFP funding resulting in the discovery of a new fumigant and replacement for methyl bromide in this reporting period.

Zhu, J., A. Dhammi, J. B. van Kretschmar, E. L. Vargo, C. S. Apperson and R. M. Roe. 2018. Novel use of aliphatic n-methyl ketones as a fumigant and alternative to methyl bromide for insect control. Pest. Manag. Sci. 74:648-657.

BACKGROUND: Fumigants like phosphine, methyl bromide and sulfuryl fluoride are highly effective for the control of urban, structural, storage and agricultural arthropod pests. Unfortunately, many of these synthetic compounds are highly toxic to people, many pests have developed resistance to these compounds, and methyl bromide, the 'gold standard' for fumigants, was de-registered because of its contribution to depletion of the stratospheric ozone layer. Alternative fumigant chemistry is needed.

RESULTS: Several plant species produce n-aliphatic methyl ketones to prevent plant herbivory. To examine the use of methyl ketones as a fumigant, structure–mortality studies were conducted using the red imported fire ant, *Solenopsis invicta* Buren, as a model. A new easy-to-use, inexpensive and disposable bioassay system was developed for this study. The LC50 values for heptanone, octanone, nonanone and undecanone were 4.27, 5.11, 5.26 and 8.21 **µ**g/cm^3 of ambient air, respectively. Although heptanone, octanone and nonanone were more effective than undecanone, subsequent research was conducted with 2-undecanone because this compound already has a US EPA registration as a biopesticide. In dose–response field studies, 12.4 mL of undecanone injected into mounds was the lowest application rate that produced no ant activity in the mound with no re-establishment of ants. Reagent grade undecanone was more cost-effective than methyl bromide for fire ants, adult German cockroaches and tobacco budworm eggs, but slightly more expensive for adult flour beetles.

Deguenon, J. M., J. Zhu, S. Denning, M. H. Reiskind, D. W. Watson and R. M. Roe. 2019. Control of filth flies, *Cochliomyia macellaria* (Diptera: Calliphoridae), *Musca domestica* (Diptera: Muscidae), and *Sarcophaga bullata* (Diptera: Sarcophagidae), using novel plant-derived methyl ketones. J. Med. Entomol. 56: 1704–1714. doi: 10.1093/jme/tjz107.

Four plant aliphatic methyl ketones were evaluated for control of adult house flies, *Musca domestica* L., blow flies, *Cochliomyia macellaria* (F.), and gray flesh flies, *Sarcophaga bullata* (Parker). In sealed petri dish assays, 2-heptanone, 2-octanone, 2-nonanone and 2-undecanone exhibited fumigant activity against house flies with 24-h LC50s of 6.9, 7.5, 8.0, and 9.2 µg/cm^3, respectively. Further research focused on undecanone (a U.S. EPA-registered biopesticide). When tested in larger enclosures at 1.7, 2.3, and 2.8 µg/cm^3, undecanone provided 60.4, 82.2, and 94.4% house fly mortality; 56.9, 75.6, and 92.5% flesh fly mortality; and 62.1, 84.5, and 97.9% blow fly mortality, respectively, after a 2-h exposure. In a two-choice behavioral assay with 194.6 µg/cm^2 of the test compound on the treatment versus an untreated surface of the same area, the overall mean repellencies for blow flies were 84.7% for undecanone versus 87.6% for N,N-diethyl-meta-toluamide (DEET). For house flies, mean repellencies were 80.7% for undecanone and 84.9% for DEET. The house fly topical LD50 for undecanone was 58.1 µg per fly. Undecanone was far less expensive for filth fly control than the gold standard for insect fumigation, methyl bromide.

6. Research progress made from earlier DWFP funding resulting in the discovery of a new, April 2023 EPA registered mosquito and tick repellent.

Roe, R. M., S. Watson and H. Beach. 2019. Insect and tick repellent formulations and methods of use thereof. Intl. Publ. No.: WO 2019/133675 A1, July 4, 2019. Intl. Appl. No.: PCT/US20 18/067625.

Roe, R. M., S. Watson and H. Beach. 2023. Insect and tick repellent formulations and methods of use thereof. US Patent Application Publication. Pub. No.: US 2023/0157278 A1, Pub. Date: May 25, 2023.

Current status. A new insect repellent was patented based on the active compound, undecanone. This was a follow-up on the project to develop this class of chemistry as a fumigant (progress discussed earlier). A NC State University Startup Company was formed, Mimikai. Angel Fund investment was used to support final product development, repellent testing, toxicology, and all costs associated with an EPA registration submission. Mimikai successfully obtained a US EPA registration in April 2023 for a final product formulation for use on skin and clothing to repel mosquitoes and ticks. The product launched is expected in the Fall 2023 worldwide.

7. Patent and licensing of a new control agent for ectoparasites including head lice.

Roe, R. M. 2019. Methods and composition for killing of insect pest eggs. Pub. No.: US 2019/0166830 A1, Jun. 6, 2019. Appl. No.: 16/321,126. PCT No.: PCT/US2017/045064.

Current status. A new head lice control system was patented and licensed to the company that has commercialized Celite and Imergard for mosquito, fly and tick control (discussed earlier in this report). One iteration of the invention is the combination of undecanone developed as a fumigant and arthropod repellent (discussed earlier) with Celite and Imergard, where the minerals enhance the activity of undecanone against head and body lice eggs. A three second exposure killed 100% of the eggs tested. This technology was licensed to the company Imerys. One unique feature of the technology, the mode of

action is non-systemic (physical), and the active, undecanone in a different formulation, is already approved by the US EPA for use on skin as a repellent (at a lower concentration of active than what is needed to kill lice); EPA registration and commercialization has the potential of being rapid for these reasons. The technology kills all stages of lice.

8. Patenting and licensing of a non-insecticidal bed net that kills mosquitoes.

Mouhamadou, C. S., R. M. Roe, R. D. Mitchell, C. S. Apperson, M. G. McCord and A. J. West. 2021. Trapping bed net for mosquito control. International Publication WO 2021/026341 A1. International Publication Date 11 February 2021. World Intellectual Property Organization International Bureau.

Mouhamadou, C. S., K. Luan, B. K. Fodjo, A. J. West, M. G. McCord, C. S. Apperson and R. M. Roe. 2020. Development of an insecticide-free trapping bed net to control mosquitoes and manage resistance in malaria vector control: A new way of thinking. 2020, 11, 732; doi:10.3390/insects11110732.

Introduction and current status. Our research with minerals to control mosquitoes was first funded by the Innovative Vector Control Consortium (IVCC) at the Liverpool, UK, School of Tropical Medicine, for applications to bed nets. Our research team was successful in functionalizing a bed net with Celite that killed greater than 80% of *Anopheles gambiae* adults after one sleep cycle in human subject testing. With funding from DWFP, this work shifted to the use of industrial minerals as a residual wall spray. Because bed nets are "stored-away" daily and washed "time-to-time", significant new technology was needed to develop a slow mineral release formulation. Although this work is worth additional study, the short-term solution was to develop a non-insecticidal, trap (T) net (with no minerals). A trapping net was developed as an alternative, that was 13 times more efficacious than the most common insecticide treated bed net used in Africa. This technology was submitted for patenting by NC State University and has been licensed to the company, Vector Textiles. Vector Textiles, is a new NCSU startup company that now has development agreements with two major bed net manufacturing companies in Africa and the US. The challenge for accessing the donor market for Africa and Asia, the WHO regulatory pathway does not recognize a non-insecticidal bed net to control mosquitoes. This restriction so far has made it difficult to attract investment to bring the T-Net product to market, limiting access to funding for Phase II and Phase III validation studies.

9. Mineral anti-microbial activity leads to discovery of a new human health risk from mosquitoes and the first discovery of the bacteria that causes scrub typhus in North America.

Deguenon, J. M., N. Travanty, J. Zhu, A. Carr, S. Denning, M. H. Reiskind, D. W. Watson, R. M. Roe and L. Ponnusamy. 2019. Exogenous and endogenous microbiomes of wild-caught *Phormia regina* (Diptera: Calliphoridae) flies from a suburban farm by 16S rRNA gene sequencing. Sci. Reports 9:20365 | https://doi.org/10.1038/s41598-019-56733-z.

Chen, K., L. Ponnusamy, C. S. Mouhamadou, B. K. Fodjo, G. C. Sadia, F. P. K. Affoue, F. P. K. AffoueI, J. M. Deguenon and R. M. Roe. 2022. Internal and external microbiota of home-caught *Anopheles coluzzii* (Diptera: Culicidae) from Coˆte d'Ivoire, Africa: Mosquitoes are filthy. PLoS ONE 17(12): e0278912. https://doi.org/10.1371/journal. pone.0278912. 24pp.

Chen, K., R. M. Roe and L. Ponnusamy. 2022. Biology, systematics, microbiome, pathogen transmission and control of chiggers (Acari: Trombiculidae, Leeuwenhoekiidae) with emphasis on the United States. Int. J. Environ. Res. Public Health 2022, 19, 15147. https://doi.org/10.3390/ijerph192215147. 24pp.

Ponnusamy, L.; R. Garshong, B. S. McLean, G. Wasserberg, L. A. Durden, D. Crossley, C. S. Apperson and R. M. Roe. 2022. *Rickettsia felis* and other Rickettsia species in chigger mites collected from wild rodents in North Carolina, USA. Microorganisms 2022, 10, 1342. https://doi.org/10.3390/microorganisms10071342. 11pp.

Richardson, E. A., R. M. Roe, C. S. Apperson and L. Ponnusamy. 2023. *Rickettsia amblyommatis* in ticks: a review of distribution, pathogenicity, and diversity. Microorganisms 2023, 11, 493. https://doi.org/10.3390/ microorganisms11020493.

Chen, K., N. V. Travanty, R. Garshong, D. Crossley, G. Wasserberg, C. S. Apperson, R. M Roe and L. Ponnusamy. 2023. Detection of *Orientia* spp. bacteria in field-collected free-living Eutrombicula chigger mites, United States. Emerging Infectious Diseases, Vol. 29, No. 8. Pp. 1676-1679.

Introduction and current status. We unexpectedly discovered anti-microbial activity for the minerals described earlier. These studies led to preliminary data that exposure of filth flies to these minerals could reduce the bacteria density on the outside of the insects and possible transfer to urban structures. The research included the development of expertise to use PCR of the 16S region of the bacteria genome and Illumina sequencing to characterize the global insect microbiome of filth flies, and development of the hypothesis that filth flies could be the vector of antibiotic resistant bacteria from animal production systems to homes and health care facilities. There is one published paper; and two additional papers written (studies in the US and Africa) not yet submitted for publication to further examine this question. Another study was conducted in Africa, which is the first to show that mosquitoes are physically moving human pathogens from the wild into homes; they are essentially acting as "filth flies". We also applied our expertise in global bacteria characterization for the first time in North America (specifically in North Carolina) to free-living chiggers and found they were infected with *Orientia* spp. bacteria, the causative agent for Scrub Typhus. The assessment of human infections in the US and that in rodents are in progress and at a late stage of evaluation, in preparation for a NIH grant proposal to continue the work in the SE US and for a DWFP or related grant proposal on chigger disease management.

Key Research Accomplishments

- Proof of concept was demonstrated (Phase I, II and III) for the use of volcanic glass, Imergard, as an indoor residual wall spray for the control of malaria mosquitoes. In Phase III trials in Africa, 4000 homes were treated and control by WHO standards was demonstrated for 11 months.
- Proof of concept was demonstrated (Phase I) for the use of volcanic glass (Imergard) and diatomaceous earth (Celite) for the control of three species of filth flies and one species of sand flies. In the case of filth flies, Phase I studies showed the minerals were more effective than for malaria mosquitoes.
- Preliminary data was obtained that Imergard and Celite are more effective on the Lyme disease tick than against mosquitoes and flies.
- Research on bed nets functionalized with Celite let to the discovery of the non-insecticidal T-Net, which in a two week Phase II field trial in Africa, was 13-fold more effective than the pyrethroid-

treated Permanet 2.0.

- The natural compound found in bananas, undecanone, was more cost effective than methyl bromide for insect control, e.g., against filth flies.
- Research with undecanone as a fumigant and the development of a novel formulation for undecanone resulted in a new commercially viable insect and tick repellent for use on skin and clothing.
- The undecanone work also resulted in a new method to control head lice eggs and other stages.
- Preliminay data on Celite showed anti-microbial activity on filth flies and led to research results suggesting antibiotic resistant bacteria might move from Ag animal production systems into homes and health care facilities.
- Filth fly antibiotic resistance research described above led to studies in Africa on the same and showed a new health impact of mosquitoes, where they physically move human pathogens from the wild into homes.
- The microbiome research described above provided an opportunity to investigate the potential of chiggers transmitting the causative agent of Scrub Typhus in the US. Our group is the first to show chiggers in NC are infected with the bacteria, *Orientia* spp., that cause the human disease, Scrub Typhus.

Reportable Outcomes

Papers Published

Deguenon, J. M., N. Travanty, J. Zhu, A. Carr, S. Denning, M. H. Reiskind, D. W. Watson, R. M. Roe and L. Ponnusamy. 2019. Exogenous and endogenous microbiomes of wild-caught *Phormia regina* (Diptera: Calliphoridae) flies from a suburban farm by 16S rRNA gene sequencing. Sci. Reports 9:20365 https://doi.org/10.1038/s41598-019-56733-z.

Deguenon, J. M., J. Zhu, S. Denning, M. H. Reiskind, D. W. Watson and R. M. Roe. 2019. Control of filth flies, *Cochliomyia macellaria* (Diptera: Calliphoridae), *Musca domestica* (Diptera: Muscidae), and *Sarcophaga bullata* (Diptera: Sarcophagidae), using novel plant-derived methyl ketones. J. Med. Entomol. 56: 1704–1714. doi: 10.1093/jme/tjz107.

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Ponnusamy, L.; R. Garshong, B. S. McLean, G. Wasserberg, L. A. Durden, D. Crossley, C. S. Apperson and R. M. Roe. 2022. *Rickettsia felis* and other Rickettsia species in chigger mites collected from wild rodents in North Carolina, USA. Microorganisms 2022, 10, 1342. https://doi.org/ 10.3390/microorganisms10071342. 11pp.

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Chen, K., J. M. Deguenon, R. D. Lawrie and R. M. Roe. 2023. Biomolecular minerals and volcanic glass bio-mimics to control adult sand flies, the vector of human Leishmania protozoan parasites. Biomolecules, 13, 1235. https://doi.org/10.3390/biom13081235.

Presentations (COVID restrictions were in effect limiting travel)

Grayson Cave, R. M. Roe and A. West. 2023. American mosquito control association 89th annual conference-Reno, NV. Joint booth with the Wilson College of Textiles displaying products created.

Marcel Deguenon, J. M., C. Riegel, M. Akogbéto, D. A. Stewart and R. M. Roe. 2022. Paradigm change for mosquito and malaria control in Africa: Imergard™ WP a non-chemical insecticide derived from volcanic glass. Oral presentation. SE US Branch Meeting of the ESA, March 26-30, 2022, San Juan, Puerto Rico.

Roe, R. M., K. Chen, J. M. Deguenon, S. Denning, R. Lawrie, M. Reiskind, W. Watson and D. Stewart. 2022. Mechanical insecticides to control mosquitoes, filth flies and sand flies. Oral presentation. SE US Branch Meeting of the ESA, March 26-30, 2022, San Juan, Puerto Rico.

Roe, R. M. 2022. Sprayable, mechanical insecticide for vector control: the future is now and more. Oral presentation. Entomological Society of American National Meeting, November 5-8, 2022, Vancouver, Canada.

Patents

Roe, R. M. 2019. Methods and composition for killing of insect pest eggs. Pub. No.: US 2019/0166830 A1, Jun. 6, 2019. Appl. No.: 16/321,126. PCT No.: PCT/US2017/045064.

Roe, R. M., S. Watson and H. Beach. 2019. Insect and tick repellent formulations and methods of use thereof. Intl. Publ. No.: WO 2019/133675 A1, July 4, 2019. Intl. Appl. No.: PCT/US20 18/067625.

Roe, R. M. 2019. Methods and composition for killing of insect pest eggs. Pub. No.: US 2019/0166830 A1, Jun. 6, 2019. Appl. No.: 16/321,126. PCT No.: PCT/US2017/045064.

Roe, R. M., S. Watson and H. Beach. 2019. Insect and tick repellent formulations and methods of use thereof. Intl. Publ. No.: WO 2019/133675 A1, July 4, 2019. Intl. Appl. No.: PCT/US20 18/067625.

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Licenses

Mimikai, new NCSU start up company. Licensed Roe's insect and tick repellent. US EPA registration granted April 2023, product launch expected Fall 2023.

Vector Textiles, new NCSU start-up company. Licensed Roe et al. non-insecticidal bed net.

Imerys, licensed Roe's head lice control system.

Imerys, Imergard and Celite licensing fee and royalties were waived since one important aim of the technology was saving lives to malaria in Africa.

Ph.D. Degrees Obtained

J. Marcel Deguenon. 2019. Evaluation of biopesticides of plant and mineral origins for insect control and role of gut microbiome in insecticide tolerance. Ph.D. in Entomology, NC State University (R. M. Roe, major professor).

Roger Lawrie. 2021. Dissertation title, Pesticide effects on gene expression: from humans to insects. Double Ph.D. in Entomology and Toxicology, NC State University (R. M. Roe, major professor)

Kaiying Chen. 2022. Dissertation title, Arthropod vector biology and human health: mechanical-mineral filth fly control, microbiota of home-caught mosquitoes and new perspective about US chiggers. Ph.D. in Entomology, NC State University (R. M. Roe, major professor).

Grayson L. Cave. 2023. Dissertation Title, Physical protection and control of agricultural and medically important arthropod pests. Ph.D. in Entomology, NC State University (R. M. Roe, major professor).

Elise Richardson. 2024. Dissertation title, Acarine control and bacterial microbiota. Ph.D. in Entomology, NC State University (R. M. Roe, major professor).

Funding Granted Resulting from this Award

Department of the Army, US Army Contracting Command, Aberdeen Proving Ground, Natick Contracting Division, Ft Detrick MD under a Deployed Warfighter Protection (DWFP) Program Grant W911SR2210005 awarded to R.M.R.

Conclusions

The research described in this report on the use of mechanical insecticides (e.g., industrial minerals) and applications that stemmed from this work to develop the first commercial, non-insecticidal bed net for killing mosquitoes should be a "wake up call" in the importance of applications in the materials sciences for the control of vector important arthropods. One major challenge is that the regulatory agencies and the regulatory processes in these agencies were developed to evaluate chemical solutions. In some cases, this is at the exclusion of mechanical methods of vector control. Traditional thinking that solutions must be chemical only in the research community must be changed. This is inhibiting innovation and blocking technology reaching the field.

This project also demonstrates how academic research can move from a university to commercial use. It is unfortunate that often those working in academic settings are not active in and not thinking about technology transfer. In some cases also, technology transfer is not valued to the level needed in the evaluation of academic performance. DWFP is an example of funding that is promoting innovation and technology transfer; this approach should be adopted at some level in other grant programs.

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Appendix (pdf of papers published) follows:

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Research

OXFORD

Control of Filth Flies, *Cochliomyia macellaria* (Diptera: Calliphoridae), *Musca domestica* (Diptera: Muscidae), and *Sarcophaga bullata* (Diptera: Sarcophagidae), Using Novel Plant-Derived Methyl Ketones

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Abstract

Filth flies live in close proximity to humans and livestock and transmit pathogens. Current control relies on chemical insecticides, and flies can develop resistance to these insecticides. The public is also interested in natural and safer insecticides. Therefore, alternative pesticides compatible with the synanthropic nature of flies are needed. Four plant aliphatic methyl ketones were evaluated for control of adult house flies, *Musca domestica* L., blow flies, *Cochliomyia macellaria* (F.), and gray flesh flies, *Sarcophaga bullata* (Parker). In sealed petri dish assays, 2-heptanone, 2-octanone, 2-nonanone, and 2-undecanone exhibited fumigant activity against house flies with 24-h LC_{50}s of 6.9, 7.5, 8.0, and 9.2 μg/cm^3, respectively. Further research focused on undecanone (a U.S. EPA-registered biopesticide). When tested in larger enclosures at 1.7, 2.3, and 2.8 μg/cm^3, undecanone provided 60.4, 82.2, and 94.4% house fly mortality; 56.9, 75.6, and 92.5% flesh fly mortality; and 62.1, 84.5, and 97.9% blow fly mortality, respectively, after a 2-h exposure. In a two-choice behavioral assay with 194.6 μg/cm^2 of the test compound on the treatment versus an untreated surface of the same area, the overall mean repellencies for blow flies were 84.7% for undecanone versus 87.6% for *N,N*-diethyl-*meta*-toluamide (DEET). For house flies, mean repellencies were 80.7% for undecanone and 84.9% for DEET. The house fly topical LD_{50} for undecanone was 58.1 μg per fly. Undecanone was far less expensive for filth fly control than the gold standard for insect fumigation, methyl bromide.

Key words: fumigant, repellent, methyl ketone, 2-undecanone, methyl bromide

Filth flies are of agricultural, medical, veterinary, and forensic importance (West 1951, Sukontason et al. 2004, Cammack et al. 2016, Nayduch 2017). Possible breeding sites for the Calliphoridae, Muscidae, and Sarcophagidae include dead animals, decaying grass, decaying or damp organic material, garbage, manure, and rotting fruits and vegetables (Hammack 1992, Nayduch and Burrus 2017). The medical and veterinary significance of filth flies has been extensively studied, as they can mechanically and biologically transmit human and animal pathogens. The common house fly, *Musca domestica*, has been associated with the transfer of more than 100 bacterial, fungal, and viral pathogens (Urzua et al. 2010, Geden 2012, Thomson et al. 2017). The secondary screwworm (SSW) or blow fly, *Cochliomyia macellaria*, and the gray flesh fly, *Sarcophaga bullata*, are also known to cause myiasis (Hagman et al. 2005, Zhu et al. 2013). Filth flies are a public health concern for people not only in the habitual environment but also in temporary living conditions including military camps (Tuck et al. 2016).

Flies are conventionally controlled with chemical insecticides, e.g., organochlorines, organophosphates, carbamates, pyrethroids, and others (Sukontason et al. 2004, Kumar et al. 2012). However, the inappropriate and wide spread use of these insecticides can be of concern to the environment and nontarget organisms including exposure to humans and animals (Sripongpun 2008, Kumar et al. 2011). Furthermore, fly chemical control has frequently caused the development of resistant filth fly strains worldwide (McKenzie and Whitten 1982, Liu and Yue 2000, Barros et al. 2001, Khan et al. 2013). It is thus important to continuously search for more effective and safer alternatives, which can also be compatible with the synanthropic nature of flies.

Plant secondary metabolism generates many compounds that are primarily used for protection against plant pathogens (Archbold

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et al. 1997) and insect pests (Wink 1988, Lee et al. 2003, Kaufman et al. 2009, Mensah et al. 2013, Rossi and Palacios 2013). Methyl ketones are ketones that contain a minimum of one methyl-carbonyl moiety, and aliphatic methyl ketones are the dominant form of methyl ketones that originate in natural processes (Forney and Markovetz 1971). These organic compounds were first described as the main constituents in the oil of common rue, *Ruta graveolens* L. (Williams 1859), and later found in the blend of volatiles emitted by various plants from several families, such as *Arecaceae*, *Cannabaceae*, *Fabaceae*, *Lauraceae*, and *Myrtaceae*, to name a few (Forney and Markovetz 1971). Their high volatility and behavior-modifying effects suggests they might be used for pest control as fumigants, repellents, or antifeeding agents (Germinara et al. 2012). Aliphatic methyl ketones are considered to be less toxic than conventional insecticides because of their rapid biodegradation in the environment, their specificity, and generally low toxicity to natural enemies, humans, and other mammals (Kaufman et al. 2009, Mann et al. 2010, Chen 2015). These products are also expected to be easier to take to market because of their already extensive use in the fragrance and flavoring industries, and because they are already registered with the U.S. EPA as a biopesticide (Mann et al. 2010).

The ketones, 2-heptanone, 2-octanone, 2-nonanone, and 2-undecanone, have been identified in various plants or plant parts including the wild tomato, *Lycopersicon hirsutum* F. *glabratum* C. H. Müll (Farrar and Kennedy 1987), Algerian oil of rue, *R. graveolens* L., essential oil of cloves, *Eugenia caryophylla* L., coconut oil, and cinnamon oil (Forney and Markovetz 1971). Undecanone has been found to be toxic to several arthropods such as the bollworm, *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae), and tobacco hornworm, *Manduca sexta* (L.) (Lepidoptera: Sphingidae) (Farrar and Kennedy 1987), German cockroach, *Blattella germanica* (L.) (Blattodea: Blattellidae) (Stumpf and Allen 2013), and twospotted spider mite, *Tetranychus urticae* Koch (Acari: Tetranychidae) (Chatzivasileiadis and Sabelis 1997). It is a U.S. EPA-registered skin-applied arthropod repellent and is the active ingredient in the commercially available arthropod repellent BioUD (HOMS LLC, Clayton, NC). BioUD was found to be more effective than *N*,*N*-diethyl-*meta*-toluamide (DEET) in repelling ticks (Witting-Bissinger et al. 2008, Bissinger et al. 2009) in filter paper assays and against mosquitoes in field trials (Witting-Bissinger et al. 2008). Few studies have investigated the fumigation activity of aliphatic methyl ketones against insects in general and especially for filth flies. Here, we investigated the fumigant properties of 2-heptanone, 2-octanone, 2-nonanone, and 2-undecanone against the common house fly, *M. domestica*, the SSW, *C. macellaria*, and the gray flesh fly, *S. bullata*. Based on these studies, we were able to conduct a cost comparison with the 'gold standard' for insect fumigants, methyl bromide. We also examined the repellency and topical toxicity of 2-undecanone against filth flies.

Materials and Methods

Chemicals

Technical grade 2-undecanone (99% purity), 2-nonanone (99%), 2-octanone (98%), and 2-heptanone (98.5%) were purchased from Sigma–Aldrich (St Louis, MO). DEET (98.1%, Jungle Juice; Sawyer Products, Inc., Safety Harbor, FL) was purchased at a local retail store and used in repellency assays.

Insects

Flies were obtained from laboratory cultures at North Carolina State University (NCSU, Raleigh, NC). *Cochliomyia macellaria* were originally field-collected at the NCSU poultry farm in Raleigh and have been in colony since 2012. The *S. bullata* colony originated from adults collected on the NCSU main campus in 2015. *Musca domestica* were from colonies maintained at Cornell University since 1982. Rearing conditions were 25 ± 2°C, 50% RH, and a photoperiod of 16:8 (L:D) h. The larval media for house flies was a mixture of Calf-Manna (MannaPro, Manna Pro Products, LLC, St. Louis, MO), distilled water, and wheat bran (The Mennel Milling Co., Fostoria, OH) at a ratio of 1:7.5:15 (v/v). *Cochliomyia macellaria* and *S. bullata* were reared as larvae on dry cat food (Purina Cat Chow Complete, Nestlé Purina PetCare Company, St. Louis, MO) that was soaked in water for 4 h at a ratio of 1.78:1 (v/v). Adults of all three species were fed a mixture of sugar and nonfat dry milk at a ratio of 3:1 and supplied with tap water ad libitum.

Petri Dish Fumigation Bioassay

The fumigant toxicity of the ketones was first evaluated under controlled laboratory conditions using Petri dishes as fumigation chambers (Zhu et al. 2018). This allowed economical dose–response studies to be conducted using minimal amounts of active and disposable test arenas. Briefly, two Petri dish bottoms (8.6 cm diameter, 1.8 cm height) were joined together to provide an inside volume of 209 cm^3. The two Petri dish halves were separated by cheese cloth (10 × 10 cm). The cloth was held on the bottom half of the chamber by a plastic O-ring. Methyl ketones in separate experiments were applied to the lower chamber (under the cloth) on a glass cover slip in different volumes ≤5 µl using a 5-µl Hamilton syringe (Part number 80016, Sigma–Aldrich) or for volumes >5 µl using a pipette (PIPETMAN P20, Gilson Inc., Middleton, WI). The insects were provided a 2-cm cotton wick saturated with a 10% sucrose solution in water placed in the upper chamber and fixed to the top surface with adhesive tape. Twenty 4- to 6-d-old adult flies, mixed sexes were used per dish. To facilitate manipulations, flies were anesthetized for 5 s with CO_2 gas at room temperature and ambient air pressure, then the flies were transferred to the upper chamber and the two Petri dish bottoms sealed together with stretched Parafilm around the seam (catalog no. S37440, Fisher Scientific, Pittsburgh, PA). The flies recovered from anesthesia in about 10 s. Structure–activity studies were conducted with 2-undecanone, 2-nonanone, 2-octanone, and 2-heptanone (v/v) for house flies only. Based on this research, subsequent Petri dish assays were conducted with 2-undecanone only for blow flies and gray flesh flies. For each species, range-finding experiments were conducted to estimate the approximate doses needed to construct probit models.

Five concentrations were used to generate each probit model. The amount of compound for each assay was determined based on its volume and weight per unit volume, assuming that no chemical escaped the sealed chamber during the exposure time. Concentrations tested were as follows: house fly (7.89, 9.47, 10.26, 15, and 16.97 µg/cm^3 of 2-undecanone; 6.67, 8.24, 9.42, 13.73, and 15.69 µg/cm^3 of 2-nonanone; 6.47, 7.84, 8.62, 13.71, and 15.67 µg/cm^3 of 2-octanone; and 6.28, 7.06, 7.85, 13.73, and 15.3 µg/cm^3 of 2-heptanone); blow fly (2.37, 3.16, 3.59, 4.34, and 6.32 µg/cm^3 of 2-undecanone); and flesh fly (7.89, 13.82, 18.15, 22.89, and 26.05 µg/cm^3 of 2-undecanone). Controls received distilled water only. These doses produced >0% and <100% mortality. The assays were run at 25 ± 3°C and 60 ± 5% RH and mortality scored at 24 h post-exposure. Experiments were carried out in four replicates, and death was defined as no movement of the insect when touched with a blunt probe.

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Small Outside Cage Experiments

To better understand the real-world use of these compounds, small cage studies were conducted with 2-undecanone and with an alternative approach to introduce undecanone into the test arena from that used for the Petri dish assays. The small cage (1.22 m each side) constructed from 5.1 by 5.1 cm lumber had an internal volume of 1.81 m^3. The frame of the cage was covered on all sides with polyethylene plastic sheeting (Model CFLWO412C, Poly-America, Grand Prairie, TX), which was replaced after each test. The undecanone was introduced using a modified airbrush dispenser (Central pneumatic quick-change airbrush kit, Harbor Freight Co., Raleigh, NC) with an air compressor (10.5 l/min, Air Admiral, model 79202, Cole Parmer Instrument Co., Vernon Hills, IL). The plastic paint cup attached to the airbrush was replaced with a graduated, screw-top glass vial to measure the amount of material delivered. A 2-cm cut was made with a razor blade through the middle of the plastic on the cage to insert the airbrush dispenser's nozzle for treatment, and the opening was resealed immediately after treatment with adhesive tape. Preliminary studies were carried out to determine the minimum concentration to provide 50% fly mortality in 2 h (not reported). For each test, 200 nonanesthetized flies of mix sexes as described before were released into the cage 15 min after the undecanone application through a new 4-cm cut made on the opposite side of the cage. To facilitate manipulations, flies were previously transferred into 50-ml falcon tubes (catalog no. 14-432-22, Fischer Scientific, Pittsburgh, PA) where the lids had been replaced with fiberglass screening (Model FCS8678-M, Saint-Gobain ADFORS, Grand Island, NY) to provide air exchange. The screen was removed and the opening of the tube inserted immediately into the cage to release the flies (the cage opening resealed afterward with adhesive tape). The delay after the undecanone application to the cage and before the flies were added allowed the undecanone time to evenly disperse by diffusion; no liquid was observed in the cage after spraying. The test compound, 2-undecanone, was diluted to 15, 20, and 25% (v/v) in ethanol (99.5% purity, Acros Organics, Fischer Scientific) in a total treatment volume of 25 ml producing concentrations of the undecanone in air in the cage of 1.71, 2.28, and 2.84 µg/cm^3. Controls received 25 ml of ethanol only. The cage was reopened 2 h post-treatment, and fly mortality was scored as previously described. Flies were collected and placed in ventilated plastic cages with sucrose, and mortality was checked again at 24 h for potential fly recovery. Experiments were performed outside in ambient sunlight at an average temperature of 30 ± 2°C and 52 ± 9% RH, and each treatment replicated in triplicate for each concentration and for each fly species. Conducting the experiments outside also prevented any accidental exposure of researchers in the laboratory.

Fumigation Cost Analysis

A cost analysis was conducted comparing 2-undecanone to methyl bromide (MeBr). MeBr is a worldwide used broad-spectrum fumigant that can effectively control a variety of pests including weeds, nematodes, fungi, snails, mites, and insects (Goodhue et al. 2005, USDA-APHIS 2016) and is the 'gold standard' for assessing new fumigants. The MeBr dosage range for treating 1 m^3 is provided in Section 18 of Fungicide and Rodenticide Act (FIFRA) of the USDA-APHIS treatment manual (USDA-APHIS 2016). The low- and high-dose range was standardized to g/m^3. The cost for MeBr in 2017 was US 0.014 $/g (Zhu et al. 2018). The treatment cost for MeBr was then estimated by multiplying the cost per gram by the recommended dosage values.

For 2-undecanone, treatment cost estimations were more challenging because its commercial cost for fumigation is not established. The undecanone wholesale price was obtained from the Alibaba web portal, and the treatment cost estimated by two methods, our direct experimental data and based on our probit models calculated from 1) the Petri dish assays and 2) small cage studies described earlier. The minimum amount of 2-undecanone to kill 100% of each fly species was determined experimentally in Petri dishes through range-finding experiments. Once the target concentration was reached, 100% morality was replicated three additional times. Twenty flies per dish were used for each replicate, and dead flies were monitored after 1, 2, and 3 d post-treatment in case there was any potential fly recovery. Mortality was scored as described earlier. For the small cage study, the highest dose tested (described earlier) against each fly species was used for the cost analysis. Treatment costs were then determined by multiplying these different amounts by the price obtained from Alibaba. The treatment cost was also calculated at the LC_{90} and LC_{99} obtained from probit models for the Petri dish and small cage studies. Labor and treatment equipment costs were not considered. In all of the above toxicity tests, the same environmental conditions and test procedures were used as described earlier but with the exceptions as just noted.

Topical Toxicity

Topical toxicity of 2-undecanone was determined using 4- to 6-d-old, unsexed adult house flies. Test concentrations (20, 10, 5, 2, and 1%) of undecanone in ethanol (same purity as described earlier) were topically applied on the dorsal thorax of each fly at 1 µl per fly using a 10-µl Hamilton syringe with repeating dispenser (PB600-1, Sigma–Aldrich). Control flies were treated with 1 µl of ethanol only. The experiment was replicated three times with 25 flies per treatment. To prevent possible fumigation effects, treated flies were placed in 2,500 cm^3 plastic cages with mesh screens on three sides to allow for as much ventilation as possible and maintained in a fume hood at room temperature and ambient light. Mortality (defined earlier) was recorded after 1 h and rechecked 18 h post-treatment.

Repellency Bioassays

The repellency tests, using 2-undecanone, were modified from the two-choice assay method used in Witting-Bissinger et al. (2008) and using the fly specking assay of Mullens et al. (2009). The test arena was a 63.6 cm^2 Petri dish bottom (8.6 cm diameter, 1.8 cm height) lined with two 31.8 cm^2 Whatman no. 1 filter paper semicircles (Whatman PLC, Maidstone, United Kingdom) held together underneath with double-sided adhesive tape. A 5-cm-diameter portion of the Petri dish lid was removed and replaced with a 6-cm-diameter fiberglass screen (Model FCS8678-M, Saint-Gobain ADFORS, Grand Island, NY) glued (Model AP 10–4, Arrow Fastener Co., LLC, Saddle Brook, NJ) to the opening made in the Petri dish lid; this allowed as much ventilation to the assay arena as possible. Undecanone was diluted to 1, 2, and 3% in 250 µl of ethanol (purity described earlier) and used to treat one filter paper semicircle giving respectively 64.85, 129.72, and 194.58 µg of active ingredient per centimeter squared. The other semicircle, the control, was treated separately to prevent cross contamination and received 250 µl of ethanol only. The two semicircles were then air dried for 5 min at room temperature and taped together to form a single filter paper disc. Ten 4- to 6-d-old adult house flies and in separate experiments blow flies, mixed sexes were briefly anesthetized with carbon dioxide as previously described and placed on the filter paper along the joining line of the two filter paper semicircles, the chamber was closed, and the flies were allowed to move freely for 3 h. Flies were

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not reused between experiments. DEET was also tested across all the treatment concentrations as described above. At the end of the exposure time, fly distribution was assessed by counting fly specks (fecal and vomit spots deposited by the flies on either the treatment or control sides of the assay chamber bottom). For both 2-undecanone and DEET, four replicates were conducted at each concentration with each replicate consisting of four choice assays in separate Petri dish arenas containing 10 flies for each choice test (160 flies total per concentration); Petri arenas were not reused. The bioassays were conducted at 24 ± 1°C and 50 ± 5% RH under ambient laboratory lighting. For both house flies and blow flies, a control was conducted with both semicircle filter papers in the repellency assay treated with the ethanol carrier only, the papers allowed to dry and then tested as described before for repellency. This control was conducted to determine whether there were any unknown environmental factors that might affect fly positioning in the test arena other than the compounds being tested (undecanone and DEET).

Statistical Analysis

Concentration–mortality response data were subjected to regression analysis by Probit (Finney 1971). Abbott's transformation (Abbott 1925) was performed as part of the probit procedure to correct for control mortality and also applied to the small cage data. The LC_{50} and LD_{50} values were considered to be significantly different if the 95% confident limits did not overlap.

To calculate percent repellency, the average percentage of specks on the control side was calculated for all replicates for each compound tested (undecanone and DEET) at each concentration examined. Same calculations were performed for the treated sides, and the data reported. A two-sided paired *t*-test was conducted to show a difference also between the treated versus the untreated surface. The proportions of specks on the control side for the three concentrations tested for each repellent were arcsine square root transformed and submitted for analysis of variance (ANOVA).

Mean mortality percentage data from the small cage studies were arcsine square root transformed and submitted for analysis of variance (ANOVA) to analyze the effect of varying concentration of 2-undecanone on fly mortality. Treatment means were compared and separated by the Student–Newman–Keuls test. Only untransformed means are reported here. All statistical analyses were performed using the R statistical software (R Foundation for Statistical Computing, Vienna, Austria; R Development Core Team 2016).

Results

Housefly Fumigation Structure–Activity Studies Using Petri Dish Bioassays

Four methyl ketones were tested as fumigants against the house fly, *Musca domestica*. The methyl ketone, 2-heptanone, had the lowest LC_{50} (6.90 µg/cm^3) followed by 2-octanone at 7.5 µg/cm^3, then 2-Nonanone at 7.96, and 2-undecanone at 9.21 µg/cm^3 (Table 1). These four compounds were chosen over other possible methyl ketones because they are found naturally in different plant species and also were available from a commercial source at high purity. Tridecanone (an aliphatic methyl ketone produced by plants) is a solid at room temperature with a low volatility rate and could not be tested as a fumigant under the conditions of our bioassay. In previous research under the same assay conditions used here for house flies, 2-heptanone was completely evaporated at the high-dose levels in 0.5 h, 2-octanone at 4 h, 2-nonanone at 10 h, and 2-undecanone at 24 h (Zhu et al. 2018). The assumption was that there was no escape of the test compounds from the fumigation chamber for the 24-h assay period, no absorption of compounds on the inner surfaces of the fumigation arena, and an even distribution of the fumigant in the gas phase. This is a reasonable assumption because no changes in the appearance of the plastic Petri plates was observed, and there was no odor of the compound outside of the bioassay.

The probit models for each methyl ketone are shown in Table 1. For all the compounds tested, there was a significant increase in mortality with an increase in concentration (2-undecanone, $F = 62.72$, df = 4, $P < 0.05$; 2-nonanone, $F = 68.34$, df = 4, $P < 0.05$; 2-octanone, $F = 60.49$, df = 4, $P < 0.05$; and 2-heptanone, $F = 53.4$, df = 4, $P < 0.05$). The best estimate of a population response is at the LC_{50}. The trend was for the LC_{50} values to decrease with carbon chain length (Table 1). However, there was no significant difference between the LC_{50} values of the four ketones tested based on 95% confidence intervals.

Blow Fly-Flesh Fly Undecanone Fumigation in Petri Dish Bioassays

Undecanone only was used to examine species variability using the Petri dish bioassay for the following reasons: there was no statistically significant difference in the LC_{50} values for the four ketones tested against the housefly (Table 1), 2-undecanone is already known and registered by EPA as an arthropod repellent with the highest potential with the least investment cost for registration as a fumigant, and undecanone was shown before to be a cost-effective alternative to MeBr for the fumigation of the red imported fire ant, *Solenopsis invicta* Buren (Hymenoptera: Formicidae) (Zhu et al. 2018). In the filth flies studies shown in Table 2, 2-undecanone was significantly more toxic to blow flies, *C. macellaria* (LC_{50} = 2.95 µg/cm^3) than for flesh flies, *S. bullata* (LC_{50} = 12.40 µg/cm^3), by a factor of 4.2. The 2-undecanone LC_{50} for house flies was 25% lower than that of the flesh fly, but the difference was not statistically significant (compare data in Tables 1 and 2) and was much higher than that of the blow fly.

Outdoor Cage Studies

An alternative assay to the Petri dish bioassay was conducted using a larger arena, a wooden frame covered with polyethylene plastic

Table 1. Lethal concentrations, LC_{50} and LC_{90}, of 2-undecanone, 2-nonanone, 2-octanone, and 2-heptanone against *Musca domestica* in Petri dish fumigation assays

Methyl ketone	*n*	Slope (SE)	LC_{50}[a] (95% CL[b])	LC_{90}[a] (95% CL[b])	χ^2
2-Undecanone	480	5.76 (0.48)	9.21 (6.95–12.31)	15.59 (9.81–24.78)	1.87
2-Nonanone	480	5.31 (0.41)	7.96 (5.85–10.95)	14.02 (8.65–22.74)	2.03
2-Octanone	480	5.37 (0.45)	7.50 (5.44–10.43)	13.47 (8.13–22.29)	2.12
2-Heptanone	480	5.47 (0.30)	6.90 (5.09–9.45)	11.95 (7.37–19.40)	2.02

[a]Values are in microgram of active ingredient per cubic centimeter. Results were Abbott corrected.

[b]In each column, lethal concentrations with overlapping confidence limits (CL) are not significantly different.

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Table 2. Lethal concentrations, LC_{50} and LC_{90}, of 2-undecanone against *Cochliomyia macellaria* and *Sarcophaga bullata* in Petri dish fumigation assays

Fly species	*n*	Slope (SE)	LC_{50}[a] (95% CL[b])	LC_{90}[a] (95% CL[b])	χ^2
C. macellaria	480	6.32 (0.45)	2.95 (2.21–3.92)	4.93 (3.15–7.76)	1.97
S. bullata	480	3.65 (0.68)	12.40 (7.86–19.56)	28.37 (14.24–56.54)	2.06

[a]Values are in microgram of active ingredient per cubic centimeter. Results were Abbott corrected.
[b]In each column, lethal concentrations with nonoverlapping confidence limits (CL) are significantly different.

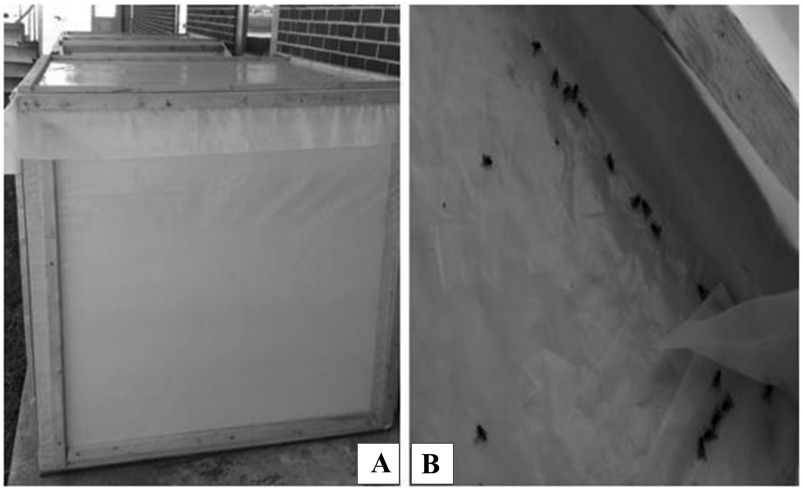


Fig. 1. (A) Fly fumigation cage. The frame was covered with polyethylene plastic on all sides which was replaced after each test. (B) Dead house flies inside cage after 2-h exposure to 2-undecanone in gas phase.

sheeting (Fig. 1). In these studies, the undecanone was applied as a spray into the cage and then time allowed for a complete transfer of the undecanone to the gas phase before the introduction of flies into the cage. This was unlike the Petri dish assays, where evaporation of the undecanone occurred over a 24-h period in the presence of the flies. Furthermore, the flies were introduced without anesthetizing them with carbon dioxide; carbon dioxide was used briefly for fly transfer to the Petri dish assay. Mortality in the small cage studies was assessed after 2 h, whereas in the Petri dish assay, the end point was 24 h. These changes in the assay method from that of the Petri dish studies were made to better approximate what might be a typical applied use of undecanone as a fumigant for insect control. The mortality end point in the small cage studies was identical to that described before. Flies were held a total of 24 h after their introduction into the cage to determine whether any recovery occurred after fly knockdown and mortality was assessed at 2 h. None of the flies that were scored as dead at 2 h had recovered at 24 h.

The cage tests (Figs. 1 and 2) essentially confirmed the results obtained from the Petri dish bioassays (Tables 1 and 2), that undecanone was an effective fumigant. The overall mean percent mortalities (mean ± SE) are presented and discussed below. For blow flies, fly mortality significantly increased as concentration increased ($F = 34.84$, df = 2, $P < 0.05$) with the three concentrations of 2-undecanone tested (1.71, 2.28, and 2.84 µg/cm^3) providing, respectively, 62.05 ± 2.54, 84.49 ± 3.31, and 97.93 ± 1.18% blow fly control in 2 h (Fig. 2A). The same trend was observed for house flies ($F = 19.09$, df = 2, $P < 0.05$) and flesh flies ($F = 25.63$, df = 2, $P < 0.05$; Figs. 2B and C, respectively). The compound, 2-undecanone, provided 60.44 ± 3.45, 82.21 ± 2.62, and 94.37 ± 3.09% house fly control (Fig. 2B) and 56.94 ± 4.53, 75.64 ± 3.27, and 92.46 ± 2.25% flesh fly control, respectively (Fig. 2C). No species difference was found in the LC_{50} values (Table 3).

Cost Comparison

Undecanone fumigation activity was measured in Petri dish bioassays and in small cage studies, and results were obtained both by direct experimental observation and based on probit model predictions. Cost assessments were conducted from these varying results. For insect pests and commodities, the minimum and maximum doses recommended by the USDA for MeBr are 40.05 and 144.17 g/m^3, respectively (USDA-APHIS 2016). The methyl bromide 2017 cost used in our analysis was 0.014 $/g (Zhu et al. 2018) and was also the bulk price in 2018 for undecanone (https://www.alibaba.com/product-detail/2-Undecanone-cas-no-112-12_60431528103.html). This cost for both methyl bromide and undecanone does not necessarily reflect the price of these actives in a final commercial product.

For the Petri dish assays with the minimum dose recommendation for MeBr and using our direct experimental data for 100% fly control, a MeBr treatment would be 5.2, 2.1 and 1.2 times more expensive than that for 2-undecanone for blow fly, house fly, and flesh fly control, respectively (Fig. 3A). At the maximum recommended dose, MeBr was 18.7, 7.7, and 4.3 times, respectively, more expensive (Fig. 3A). Similar results were obtained from the probit models. At the minimum MeBr dose and at the LC_{99} for undecanone, MeBr was 5.3 and 1.7 times more expensive to control blow flies and house flies, but 1.39 times less expensive for flesh flies. At the maximum dose, MeBr was 19.0, 6.0, and 2.6 times more expensive (Fig. 3B).

The cost difference for undecanone versus MeBr observed in the Petri dish bioassays was even greater for the small cage studies

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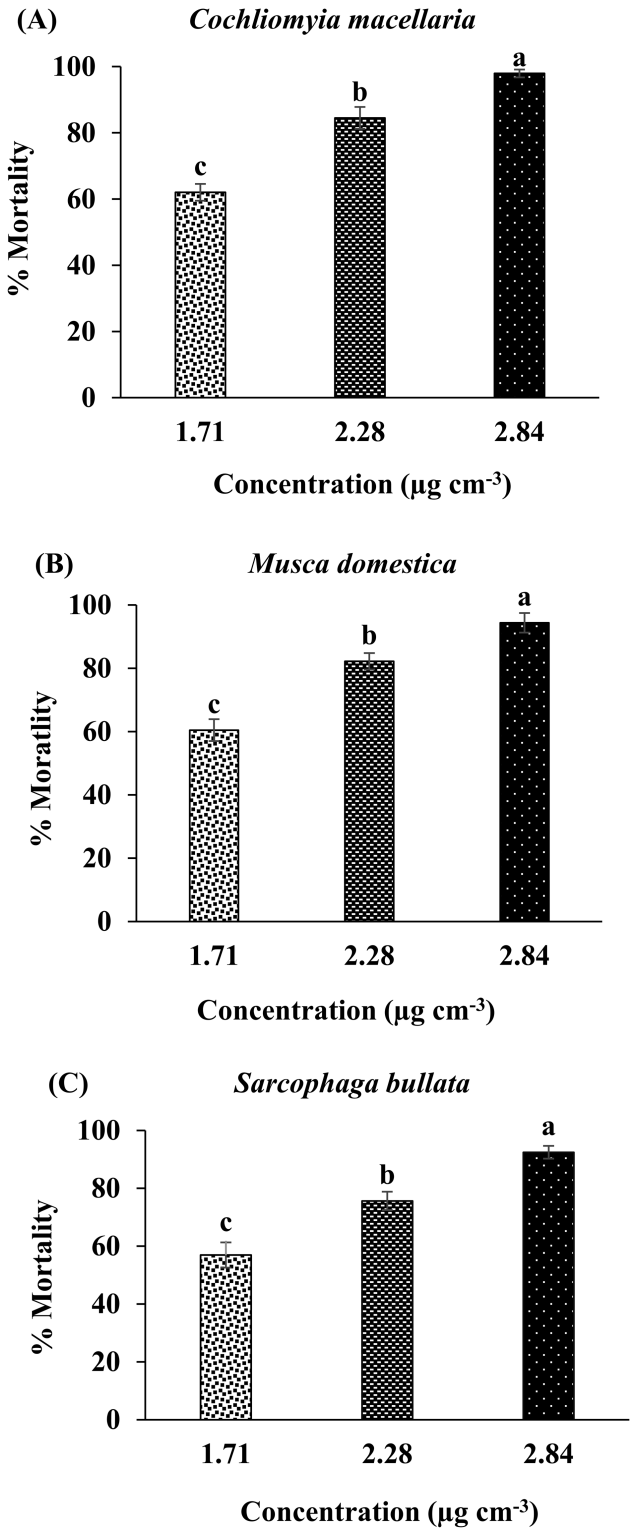


Fig. 2. Percent mortality of different filth fly species exposed to 2-undecanone in small (field) cage experiments: (A) *Cochliomyia macellaria*, (B) *Musca domestica*, and (C) *Sarcophaga bullata*. Concentrations are in micrograms of active ingredient per cubic centimeter, and percentage mortality was Abbott corrected. Bars indicate mean mortality values of untransformed data ± SE. Bars with different letters differ significantly ($P < 0.05$, Student–Newman–Keuls) in their mortality rates.

(due to the lower amounts of undecanone needed to produce higher mortality rates in the small cage arena). Based on the highest dose of undecanone that was tested, MeBr at its minimum dose recommended would be 14 times more than the cost of undecanone for 97% blow fly, 94% house fly, and 92% flesh fly control. With the high MeBr dose, the cost would be at least 50 times more than undecanone (Fig. 4A). The same trend was found using the Probit predictions at the LC_{90} and LC_{99}, with undecanone being much lower in cost than methyl bromide (Fig. 4B). Zhu et al. (2018) found similar results when examining the use of undecanone for red imported fire ant control, regardless of the recommended dose (minimum or maximum); 2-undecanone was more cost-effective than MeBr not only to the red imported fire ant but also to the German cockroach and the tobacco budworm.

Topical Toxicity

Fumigation of flies presents the toxicant topically in the gas form to the insect surface and also potentially through respiration. To better understand the role of topical exposure, house flies were treated with 2-undecanone at different concentrations in ethanol by direct application to the insect thorax. The probit model as well as concentrations that produced 50 and 90% mortality are presented in Fig. 5. The LD_{50} and LD_{90} were 58.1 and 310.02 µg per fly, respectively.

2-Undecanone as a Repellent for Filth Flies

Undecanone has been commercialized as a mosquito and tick repellent (Innocent et al. 2008, Witting-Bissinger et al. 2008, Bissinger et al. 2009). Research here is the first time this methyl ketone has been evaluated as a repellent for filth flies. Because flies treated by fumigation could be repelled from a treated area, repellency could be an important factor in the practical use of undecanone as a fumigant. Blow flies, *C. macellaria*, and house flies, *Musca domestica*, were the most susceptible flies in fumigation assays and were the flies tested for repellency. The appearance of fly specks in a two-choice assay (treated vs control surface; Fig. 6) was used to evaluate fly location over time (Axtell 1970, Mullens et al. 2009). The appearance of greater specks on the control surface was a measure of greater repellency from the treated surface. In these studies, the test arena was designed to reduce possible fumigation, but fumigation effects could not be completely eliminated. Because fumigation from the treated surface would be expected to be highest when compared with the control side, fumigation effects resulting in toxicity and reduce fly mobility would probably result in an under representation of true percentage repellency. General observations during the study suggested that the majority of the flies were on the control sides as shown in Fig. 5. No significant difference was found when blow flies ($t = -0.056$, df = 3, $P = 0.959$) or house flies ($t = -0.458$, df = 3, $P = 0.678$) were given a choice between two untreated surfaces. This would be expected when there are no external environmental cues affecting fly choice. Mean percentage of specks (± SE) using 2-undecanone and DEET separately are presented in Tables 4 and 5.

Musca domestica deposited significantly more specks on the control compared with the treatment when exposed to 1% ($t = -3.764$, df = 3, $P = 0.033$), 2% ($t = -11.334$, df = 3, $P = 0.001$), and 3% ($t = -7.3619$, df = 3, $P = 0.002$) undecanone. The overall mean repellencies (± SE) for undecanone at these concentrations were 63.43 ± 3.49, 72.79 ± 1.92, and 80.67 ± 2.46%, respectively (Table 4). DEET provided similar results on house flies; significantly more specks were deposited on the controls at 1% ($t = -4.710$, df = 3, $P = 0.020$), 2% ($t = -7.734$, df = 3, $P = 0.004$), and 3% ($t = -30.283$, df = 3, $P < 0.001$). Mean repellencies (± SE) for DEET were 64.22 ± 2.97, 77.08 ± 2.18, and 84.89 ± 1.27% (Table 4). Also, significantly more specks were left on the controls as the concentration of 2-undecanone ($F = -7.423$, df = 2, $P < 0.05$) and DEET ($F = 17.68$, df = 2, $P < 0.05$) increased.

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Table 3. Concentrations of 2-undecanone that produced 50, 90, and 99% fly mortality (predicted by probit model) and 92–100% fly mortality (by direct observation) in fumigation studies in Petri dish assays and small cage studies

Bioassay	Target pest	Dose (g/m³)[a]			
		Calculated			Direct observation (% Mortality)[c]
		LC_{50} (95% CL[b])	LC_{90} (95% CL[b])	LC_{99} (95% CL[b])	
Petri dish[d]	*C. macellaria*	2.95 (2.21–3.92)	4.93 (3.15–7.76)	7.54 (3.33–17.10)	7.7 (100)
	M. domestica	9.21 (6.95–12.31)	15.59 (9.81–24.78)	23.96 (10.20–55.9)	18.78 (100)
	S. bullata	12.4 (7.86–19.56)	28.37 (14.24–56.6)	55.73 (15.6–199.1)	33.45 (100)
Fly cage[e]	*C. macellaria*	1.56 (0.89–2.77)	2.38 (1.52–3.72)	3.35 (1.28–8.75)	2.84 (97)
	M. domestica	1.55 (0.78–3.09)	2.57 (1.45–4.53)	3.88 (1.08–13.96)	2.84 (94)
	S. bullata	1.62 (0.83–3.14)	2.78 (1.42–5.47)	4.36 (1.99–19.05)	2.84 (92)

[a]Calculated doses were obtained from probit models, whereas the direct observation doses were obtained from empirical results.
[b]Confidence limits.
[c]Original data for highest dose used in the fly cage studies were presented in Materials and Methods section; percentage mortality for each also shown in Fig. 2.
[d]LC_{50} and LC_{90} data are presented here for comparison purposes only. The original data along with the probit model statistics were presented in Tables 1 and 2.
[e]The probit model statistics are as follows: for *C. macellaria*, n = 2,400, slope (SE) = 7.04 (0.27), χ^2 = 1.01; for *M. domestica*, n = 2,400, slope (SE) = 5.83 (0.31), χ^2 = 1.05; and for *S. bullata*, n = 2,400, slope (SE) = 5.41 (0.32), χ^2 = 0.99.

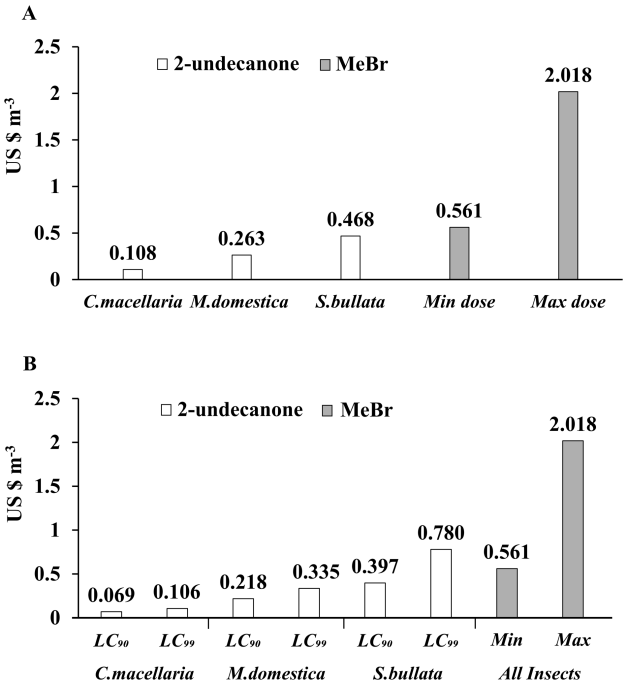


Fig. 3. Cost comparison of 2-undecanone versus methyl bromide as a fumigant for fly control based on Petri dish bioassays compared with the minimum (40.05 g/m³) and maximum (144.17 g/m³) USDA recommended MeBr doses for insects. (A) Results were based on concentrations that produced 100% mortality for each fly species (shown in Table 3); these concentrations of 2-undecanone were obtained by direct observation using the Petri dish bioassay described in the methods. (B) Results were based on concentrations that produced 90 and 99% mortalities (shown in Table 3); these concentrations of 2-undecanone were obtained by probit analysis.

Cochliomyia macellaria significantly avoided the filter papers treated with 2-undecanone and deposited more specks on the control sides at 1% (t = –3.3667, df = 3, P = 0.036), 2% (t = –9.8336, df = 3, P = 0.001), and 3% (t = –10.76, df = 3, P = 0.001). The overall mean repellencies (± SE) for undecanone at these concentrations were 62.92 ± 3.77, 75.29 ± 2.04, and 84.71 ± 3.52% (Table 5). DEET again provided similar results with the controls having significantly more specks at 1% (t = –7.301, df = 3, P = 0.005), 2% (t = –12.584, df = 3, P = 0.001) and 3% (t = –16.726, df = 3, P < 0.001). Mean repellencies (± SE) for DEET were 68.20 ± 2.38, 80.06 ± 2.12, and 87.57 ± 1.73% (Table 5). As concentrations of 2-undecanone (F = 11.56, df = 2, P < 0.05) and DEET (F = 21.68, df = 2, P < 0.05) increased, blow fly repellency also significantly increased.

Discussion

Filth Fly Fumigation Activity of Aliphatic Methyl Ketones

All of the aliphatic methyl ketones examined were active against house fly, *M. domestica*, adults in the petri dish assay. The trend was for the house fly fumigation activity to increase with shorter chain length although the differences between compounds were not statistically significant (Table 1). Using the same assay method and methyl ketones, an identical trend was found between carbon chain length and fumigation activity with the adult red imported fire ant, *S. invicta* (Zhu et al. 2018; in the ant studies, some of the differences were statistically significant). Zhu et al. (2018) also found the volatility of the methyl ketones increased from C-11 to C-7 (discussed earlier). Therefore, the *M. domestica* toxicity trend (a lower LC_{50}; Table 1) was positively correlated with a decrease in carbon chain length and positively correlated with increased volatility. Because we examined mortality at 24 h and the fumigant concentration by weight in the vapor phase was higher earlier for the shorter methyl ketones, we were unable to determine what impact volatility alone had on toxicity against the house fly. It is possible that the lower molecular mass methyl ketones are more toxic to house flies. However, it is also possible that because of the higher evaporation rate, the time of exposure of the full dose in the gas phase was longer as the carbon chain is reduced, and this was affecting the measured susceptibility. There could also be a combination of effects based on toxicity differences between compounds and time to full evaporation. Interestingly, at the LC_{50}, the number of moles per cubic centimeter for 2-undecanone, 2-nonanone, 2-octanone, and 2-heptanone were almost the same, i.e., 5.4 × 10^{-2}, 5.6 × 10^{-2}, 5.9 × 10^{-2}, and 6.0 × 10^{-2}, respectively, which is another factor to consider in evaluating toxic action. Outdoor large cage studies are discussed later where the impact of time for evaporation on toxicity was eliminated as a factor. Methyl ketone species differences in fly susceptibility in the

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Petri dish assay was conducted using only undecanone. It was clear that the blow fly, *C. macellaria*, was more susceptible with a LC_{50} of 2.95 µg/cm³ versus that for the house fly and flesh fly, *S. bullata*, i.e., 9.2 and 12.4, respectively.

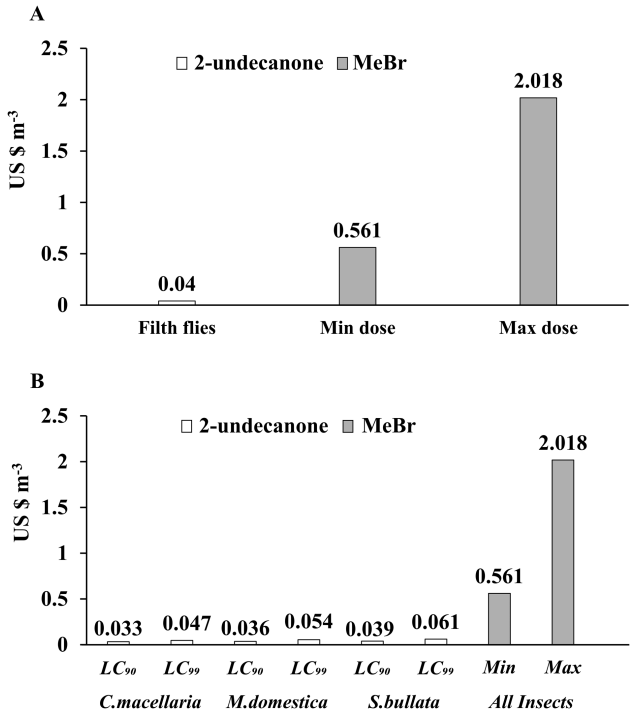


Fig. 4. Cost comparison of 2-undecanone versus methyl bromide as a fumigant for fly control in small cage studies and using the minimum (40.05 g/m³) and maximum (144.17 g/m³) recommended doses for MeBr by the USDA for insect control. (A) Results were based on the concentration of 2-undecanone (2.84 g/m³) that produced 92, 94, and 97% mortality of *Sarcophaga bullata*, *Musca domestica*, and *Cochliomyia macellaria*, respectively (shown in Table 3); these undecanone concentrations were obtained by direct observation using the bioassay architecture shown in Fig. 1 and methods described in the Materials and Methods section. (**B**) Results were based on concentrations that produced 90 and 99% mortality (shown in Table 3); these concentrations of 2-undecanone were obtained by probit analysis.

An alternative assay to the Petri dish bioassay was conducted using a larger arena (Fig. 1) to better approximate a practical application. In these studies, the undecanone was applied as a spray and allowed to fully evaporate before the introduction of flies. This was unlike the Petri dish assays, where evaporation of the undecanone occurred over a 24-h period in the presence of the flies. Furthermore, the flies were introduced without anesthetizing them with carbon dioxide, unlike the Petri dish assay, and mortality was assessed after 2 h versus 24 h for the Petri dish studies.

The cage tests (Figs. 1 and 2) essentially confirmed the results obtained from the Petri dish bioassays (Tables 1 and 2) that undecanone was an effective fumigant. No insects moved when touched with a blunt probe at 2 h and also did not move after 24 h. These results suggest that knockdown and death occurred rapidly. Further studies are needed to determine when knockdown versus mortality occurred prior to 2 h. From a practical perspective for fly control, the rapid knockdown and death permits a shorter waiting period after treatment for ventilation and reentry into the treated area. Another advantage of undecanone, because of its high volatility compared with many insecticides, residual activity after ventilation is not expected.

The other interesting discovery was the higher activity of 2-undecanone in the cage studies versus the Petri dish assays (Table 3). For example, there is a 5.9-fold statistically higher LC_{50} for house flies for the Petri dish assay versus the small cage study. The LC_{50} for flesh flies in Petri dish assays was 7.6 times significantly higher than that of the cage studies. We do not understand the reason for the higher activity of undecanone in the small cage studies. Possible explanations could include complete evaporation of the undecanone at the start of the treatment (different from the Petri dish assay), the carbon dioxide used in the Petri dish assay to anesthetize the flies may have reduced their susceptibility to methyl ketones, and/or the ethanol carrier used in the cage studies may have increased the activity of undecanone (ethanol alone was not toxic to the flies).

Zhu et al. (2018) suggested that undecanone had a physical mode of action disrupting cuticular lipids leading to water loss and death. It is well known that volume increases by the cube, whereas surface area by the square as insect size increases. This physical relationship increases the impact of water evaporation from an insect's cuticle as the insect size decreases. Adults of the flesh flies on average weighed 77.5 mg as

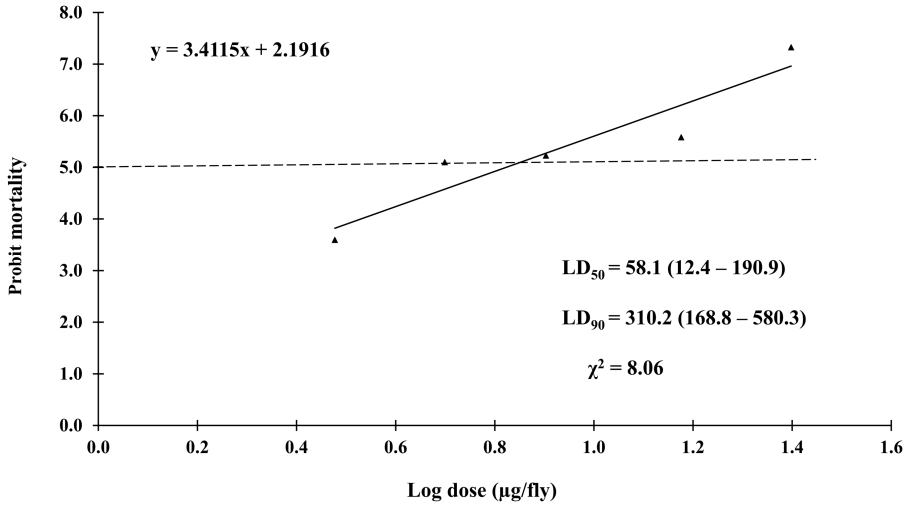


Fig. 5. Probit model for log dose versus probit mortality for the topical application of 2-undecanone on the thorax of adult house flies, *M. domestica*. The lethal doses, LD_{50} and LD_{90}, as well as the confidence limits (in parentheses) are in microgram of active ingredient per fly. The small triangles in bold represent the concentrations tested, and the dotted line represents 50% mortality.

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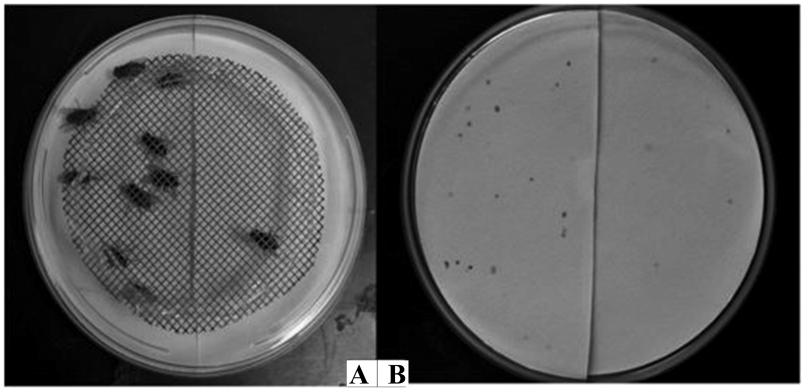


Fig. 6. Example of repellency bioassay (see Materials and Methods for details), left side treated with ethanol carrier, right side with 2-undecanone in ethanol. (A) Fly distribution during test. (B) Specks are from regurgitation and/or defecation. The greater number of specks on the control (left side) is a function of fly time on surface (the more specks on a particular half circle, the longer the flies were on that surface) and a measure of repellency from the treated surface (right side).

Table 4. Percentage of specks deposited by *Musca domestica* on treated and control surfaces in Petri dish repellency assays

Chemical	Concentration (µg/cm²)	Percentage of specks on treated surface ± SE (%)[a]	Percentage of specks on control surface ± SE (%)[a] (=% repellency)	*P* values[b]
2-Undecanone	64.85	36.57 ± 3.49	63.43 ± 3.49	0.033
	129.72	27.21 ± 1.92	72.79 ± 1.92	0.001
	194.58	19.33 ± 2.46	80.67 ± 2.46	0.002
DEET	64.85	35.78 ± 2.97	64.22 ± 2.97	0.020
	129.72	22.92 ± 2.18	77.08 ± 2.18	0.004
	194.58	15.11 ± 1.27	84.89 ± 1.27	<0.001

DEET (*N*,*N*-diethyl-*meta*-toluamide).
[a]Percentage values were obtained from untransformed data.
[b]*P* values were obtained from two-sided paired *t*-tests.

Table 5. Percentage of specks deposited by *Cochliomyia macellaria* on treated and control surfaces in Petri dish repellency assays

Chemical	Concentration (µg/cm²)	Percentage of specks on treated surface ± SE (%)[a]	Percentage of specks on control surface ± SE (%)[a] (=% repellency)	*P* values[b]
2-Undecanone	64.85	37.08 ± 3.77	62.92 ± 3.77	0.036
	129.72	24.71 ± 2.04	75.29 ± 2.04	0.001
	194.58	15.29 ± 3.52	84.71 ± 3.52	0.001
DEET	64.85	31.80 ± 2.38	68.20 ± 2.38	0.005
	129.72	19.94 ± 2.12	80.06 ± 2.12	0.001
	194.58	12.43 ± 1.73	87.57 ± 1.73	<0.001

DEET (*N*,*N*-diethyl-*meta*-toluamide).
[a]Percentage values represent untransformed data.
[b]*P* values were obtained from two-sided paired *t*-tests.

compared with blow flies and house flies at 20.1 and 15.8 mg per fly, respectively. Yet house flies and flesh flies greatly different in size, had the same LC_{50}. So, the species differences in susceptibility to undecanone fumigation was not related to insect size. Additional studies are needed to better understand the mode of action of methyl ketones as an insect fumigant. The species differences noted here might provide a mechanism to better understand undecanone action.

The fumigant property of plant extracts and essential oils on insects was studied before in filth flies (Lee et al. 2003, Urzua et al. 2010, Kumar et al. 2011, Rossi and Palacios 2013, Bedini et al. 2017), but almost no work was conducted on individual components in these mixtures and in particular methyl ketones. This is the first study of methyl ketones used as a stand-alone fumigant against filth flies. Short-chain aliphatic methyl ketones showed fumigant toxicity against adults of the granary weevil, *Sitophilus granarius* (L.) (Coleoptera: Curculionidae), under laboratory conditions (Germinara et al. 2012). Zhu et al. (2018) also successfully demonstrated the use of undecanone as a fumigant for red imported fire ant adults, German cockroach adults, red flour beetle adults, *Tenebrionidae castaneum* (Herbst) (Coleoptera: Tenebrionidae), and the tobacco budworm caterpillar, *Heliothis virescens* (F.) (Lepidoptera: Noctuidae). The research of Zhu et al. (2018) was translational in that a practical field use for undecanone was demonstrated for fire ant mound control.

Undecanone as an Alternative for Methyl Bromide

As an ozone-depleting substance, MeBr has been scheduled for complete phase out for 2005 in developed countries and 2015 in developing countries under the Montreal Protocol. However, quarantine and pre-shipment uses are not included in the phase out, and the

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Protocol also provides the possibility for Critical Use Exemptions after the phase out if no technically and economically feasible alternative to MeBr exists (Goodhue et al. 2005, USDA-APHIS 2016). Also, under Section 18 of the U.S. Federal Insecticide, FIFRA, which allows the use of nonregistered pesticides under 'emergency conditions', a list of commodities can be treated with MeBr until March 2020 (USDA-APHIS 2016). Furthermore, some of the very limited compounds approved to be used as potential alternatives such as sulfuryl fluoride do not provide consistent results or are not cost-effective (USDA-ERS 2000, Adam et al. 2010, Hong 2014). It is therefore important to continue to search for effective alternatives. The cost-effectiveness of 2-undecanone as a fumigant was investigated before for agricultural pests (fire ants, caterpillars, beetles, and cockroaches, Zhu et al. 2018) and is under investigation here for U.S. military operations, as a possible public use for filth flies, and as a possible replacement for MeBr.

Because MeBr has been the 'gold standard' for fumigants, it appeared reasonable to compare the price of undecanone with that of MeBr. It was clear from the cost comparisons in this study for filth flies for both the Petri dish bioassays and the outdoor cage studies (which better approximated the practical use of a methyl ketone as a fumigant), undecanone had a lower cost for insect control than MeBr, in some cases, a 50-fold lower cost to obtain complete control (discussed in more detail earlier; also see Figs. 3 and 4). Zhu et al. (2018) found similar results when examining the use of undecanone for red imported fire ant control, regardless of the recommended MeBr dose (minimum or maximum). Zhu et al. (2018) found that 2-undecanone was more cost-effective than MeBr not only to the red imported fire ant but also to the German cockroach and the tobacco budworm. This cost-effectiveness of 2-undecanone against a variety of insects (as also shown in this paper) and plants (reviewed by Zhu et al. 2018), along with its low nontarget toxicity, makes it a good candidate as an alternative to MeBr.

Topical Toxicity and Repellency of Undecanone

The LD_{50} for house flies in this study (58.1 µg per fly) was lower than that of leaf oils from *Matricaria chamomilla* L. and *Clerodendron inerme* G. (76 and 84 µg per fly) applied topically on *M. domestica* (Shoukry 1997). It was also lower than the LD_{50} of eucalyptol (118 µg per fly in males and 177 µg per fly in females; West 1951) or the LD_{50}s of most monoterpenoids tested (20 out of 22 tested) by Rice and Coats (1994) on *M. domestica*, which averaged around 200 µg per fly. In contrast, the LD_{50} of 2-undecanone was higher than that of several essential oils (23 out of 34 evaluated) extracted from plants against house flies (Pavela 2008). Undecanone applied topically provided over 50% mortality in 1 h as flies recorded as dead after 1 h did not recover 18 h post-treatment. These results suggest that 2-undecanone could be used as a contact insecticide to control filth flies. In these topical studies, the flies were well ventilated to reduce fumigation effects as much as possible, but there was no way to determine the levels of undecanone in the vapor form near the fly surface.

Our studies also clearly show that 2-undecanone repels flies even at the lowest concentration of 1%. This was expected because plant secondary metabolites have been shown before to repel various dipteran species of medical and veterinary importance including blow flies (Bedini et al. 2017), house flies (Zhu et al. 2009, Kumar et al. 2011), stable flies (Zhu et al. 2009, 2012; Hieu et al. 2010), and sand flies (Yaghoobi-Ershadi et al. 2006). At all concentrations and regardless of the fly species, filth flies avoided 2-undecanone- and DEET-treated surfaces at comparable rates (Tables 4 and 5). Witting-Bissinger et al. (2008) in arm-in-cage studies found no differences in mean repellency over 6 h after application for BioUD containing 7.75% undecanone and for 7% DEET for the yellow fever mosquito, *Aedes aegypti* (L.) (Diptera: Culicidae), and the Asian tiger mosquito, *Aedes albopictus* (Skuse) (Diptera: Culicidae). Plant secondary metabolites also have both fumigant and repellent activities; e.g., several aliphatic ketones such as 2-pentanone, 2-hexanone, 2,3-butanedione, and even 2-heptanone showed fumigant toxicity against adults of the granary weevil, *S. granarius* (Germinara et al. 2012), which were also repelled (Germinara et al. 2008).

In conclusion, this study investigated the bioactivity of methyl ketones against three species of filth flies of medical and veterinary importance. The findings from this study coupled with other research (Germinara et al. 2012), including the one previously published from our laboratory on the red imported fire ant, German cockroach, red flour beetles, and tobacco budworm (Zhu et al. 2018), indicate that the methyl ketone 2-undecanone is a potential fumigant. Furthermore, it is more cost-effective than methyl bromide. Because 2-undecanone is also active as a killing agent on contact with flies and a fly repellent, additional uses of the technology are available other than fumigation.

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Exogenous and endogenous microbiomes of wild-caught *Phormia regina* (Diptera: Calliphoridae) flies from a suburban farm by 16S rRNA gene sequencing

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The black blow fly, *Phormia regina* (Meigen) (Diptera: Calliphoridae) is one of the most abundant carrion flies in North America. Calliphorids are important in agriculture and animal production, veterinary sciences, forensics and medical entomology. While the role of flies in the epidemiology of human and animal diseases is an active area of research, little is known about the microorganisms associated with these insects. We examined the diversity of wild-caught black blow fly endogenous (internal body) and exogenous (external body) microbial communities using 16S rRNA gene sequencing. Overall, 27 phyla, 171 families and 533 genera were detected, and diversity was significantly higher ($P < 0.05$) on external body surfaces. At the genus level, *Dysgonomonas*, *Ignatzschineria*, *Acinetobacter*, *Vagococcus*, *Myroides*, and *Wohlfahrtiimonas* were predominant. Cloning and sequencing of nearly full-length fragments of the 16S rRNA gene showed that some of the species identified are known to be pathogenic to humans, animals, and plants. *Myroides odoratimimus* and *Acinetobacter radioresistens* are well-known, multi-drug resistant bacteria. These results provide a snapshot of the microbial communities harbored by adult black blow flies and call for more comprehensive studies to better characterize the role these flies may play in the transmission of pathogenic microorganisms.

Blow flies (Diptera: Calliphoridae) are a group of medium to large flies with a worldwide distribution, usually harboring bright or metallic colors[1]. In calyptrate flies, calliphorids represent about 1,500 described species and account for 8% of species diversity[2,3]. They are saprophagous/necrophagous insects, breeding in decaying organic matter of plant and animal origins and are of medical[4–6], veterinary/agricultural[7], and forensic importance[8,9]. For example, adult calliphorids are known to be the first insects to colonize animal and human remains and are frequently used in the estimation of the minimum postmortem interval (minPMI) in medico-legal investigations[10]. Several species of blow flies, such as the primary screwworm, *Cochliomyia hominivorax* (Coquerel) and the Australian sheep blow fly, *Lucilia cuprina* (Wiedmann) (Diptera: Calliphoridae), are notorious myiasis (fly-strike) agents in livestock causing hundreds of millions of dollars in economic losses[7,11].

The presence of blow flies in microbe-rich substrates, such as manure, carrion and corpses, confer them a high probability of encountering/interacting with a large variety of microorganisms such as bacteria[12], while their synanthropic nature (association with human environments) as well as strong flight abilities[13], allow them to potentially disseminate those organisms over long distances into human habitat; they have the potential of infecting different substrates through contact by just landing on a surface but also by regurgitation of their gut content and defecation[14–16]. In addition, it has also been shown that blow flies can carry and mechanically transmit several bacterial pathogens, including *Burkholderia pseudomallei*[17], *Providencia rettgeri*, *Morganella morganii*, *Klebsiella* spp. and *Staphylococcus aureus*[18]; *Escherichia coli*[19,20]; and *Helicobacter pylori*[21].

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The black blow fly, *Phormia regina* (Meigen) (Diptera: Calliphoridae), the focus of this paper, is a Holarctic fly and is distributed throughout the Northern Hemisphere[22]. In the USA, *P. regina* is typically a cold weather fly, as it is more abundant during the "chilled" summer months in the Northern part of the US while being more frequent in the cooler months of spring and fall in the South[23,24]. However, high population densities of *P. regina* have previously been recorded during the summer in Kansas[25] and California[26]. *Phormia regina* has been of interest since it is one of the most abundant carrion flies in North America[27] and is one of the primary insects used to estimate the minimum postmortem interval (minPMI) in forensic entomology[28]. *Phormia regina* is also listed by the U.S. Food and Drug Administration (FDA) as one of the 22 pests (the "Dirty 22") that could potentially spread food-borne pathogens[29] and is an important research model for the study of fly physiology[30–32]. Several studies have been conducted on *P. regina* morphology[33], genomics[27], biology and ecology[9,34–36], including aspects of the interactions that exist between this species and bacteria[37]. However, studies that investigated the microbial communities in free-flying, wild-type adult *P. regina* using 16S rRNA gene sequencing are absent. Weatherbee *et al.*[37] investigated the internal microbiome of *P. regina* collected from swine carcasses but focused on third-stadium larvae. Russell *et al.*[38] investigated the presence of antibiotic-resistant bacteria in adult Calliphorids collected from piglet cadavers introduced into a forest environment but only identified the flies to the family level. Also, the study was based on culture-dependent techniques.

In the present study, we investigated the endogenous and exogenous bacterial microbiomes of wild-caught adult black blow flies collected in a suburban environment using high-throughput 16S rRNA gene amplicon sequencing. We hypothesized that (i) the microbial communities from these two regions of the fly body would be different, (ii) bacterial diversity on the outside will be higher compared to the gut and (iii) the flies collected will harbor known pathogenic bacteria.

Material and Methods

Fly collection, identification, and initial sample preparation. Adult blow flies (mixed sexes) were collected at the Lake Wheeler Road experiment station at NC State University (NCSU), Raleigh, North Carolina in November 2017. This field station contains several animal production systems including a feed mill laboratory, equine education center, poultry waste management facility, etc.) and plant-related units (an agroecology farm, soil & water research center, etc.). This experiment station is located close to the main NCSU campus and urban areas in the city. Blow flies are strong fliers and highly adapted to evasion making it difficult to collect them individually in flight or after they have landed, therefore, an effective alternative approach is trapping. Flies were collected at a dump site (35°43′50.54″N, 78°42′13.52″W) using a Captivator fly trap jug (Starbar Products, Schaumburg, IL, USA) that contained 900 ml of a Flies-Be-Gone fly attractant solution (Flies-Be-Gone, Toms River, NJ, USA) (Fig. S1). The jug was covered with window screening (Model FCS8678-M, Saint-Gobain ADFORS, Grand Island, NY, USA) to prevent fly access to the attractant solution, and the trap was placed beneath a partially screened pyramid. The 1.2 m × 1.2 m × 1.2 m pyramid was constructed with standard framing lumber, and the screen was attached to the upper half with staples. Attracted flies that entered the pyramid were trapped at the top in an inverted 1.9 L Rubbermaid food storage canister (Model #007420216, Walmart, Raleigh, NC, USA). To prevent contamination of the flies from the trapping container itself, this container was washed with detergent, rinsed with sterile water and then cleaned with 95% ethanol. To reduce possible fly-fly interactions and fly-trap-fly interactions from affecting the fly microbiome, flies were collected only one hour after placement of the fly trap and transferred to the laboratory immediately. Flies were placed individually in 1.5 ml sterile microcentrifuge tubes (USA Scientific, Ocala, FL, USA), and the surface microbiota was collected by rinsing each fly 5 times, each time in 200 µl of sterile phosphate-buffered saline (PBS, Cat #00-3000, Invitrogen, MD, USA). All washes per fly were pooled into a 2 mL sterile microcentrifuge tube. The pooled surface washes (per fly) as well as the adult flies were then stored at −40 °C until further use. The flies were identified based on morphological characteristics[39].

DNA extraction and quantification. Prior to endogenous microbial DNA extraction, each adult fly was surface sterilized using 70% ethanol (30 s) followed by 1% bleach in water (30 s) and then washed 5 times with sterile water. The final washes were pooled together for subsequent verification of sterility. DNA extraction from internal (12 endogenous samples) and external body (12 exogenous samples) extracts was modified from Ponnusamy *et al.*[40]. Briefly, the pooled exogenous PBS wash (1 mL) was lyophilized and reconstituted with 200 µL of TNE buffer. For endogenous sampling of the microbiome, each fly was transferred to a sterilized screw cap 2 mL microcentrifuge tube (Catalog # 02-681-344, Fisher Scientific, Pittsburgh, PA, USA) containing 10 sterilized 3 mm solid glass beads (Catalog #11-312 A, Fisher Scientific, Pittsburgh, PA, USA) each and was homogenized in 200 µL TNE buffer using the FastPrep FP120 system (Thermo Electron Corporation, Waltham, MA, USA) for 30 s. For lysis, we added 160 µL of lysis buffer 1 (TNE buffer [100 mM Tris, 0.2 M NaCl, 10 mM EDTA, pH 7.4] containing 20 µL of proteinase K and 20 µl lysozyme). The samples were then incubated at 37 °C for 1 h. After which 200 µl of lysis buffer 2 (1% cetyltrimethylammonium bromide [CTAB], 1.5 M NaCl, 0.5 M Tris-HCl [pH 8.0], 0.1 M EDTA [pH 8.0]) was added, and the samples were further incubated at 56 °C for 1 h. The use of the whole body to sample the endogenous microbiome allowed us to account for bacteria present internally but localized to organs other than the gut. DNA was isolated through phenol-chloroform extraction and ethanol precipitation, and the resulting DNA was resuspended in 100 µl of molecular grade water. The DNA samples were further purified using the Wizard DNA cleanup system (Promega, Madison, WI, USA). The DNA quality and quantity were assessed using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The total genomic DNA was normalized to 50-100 ng/µl and stored at −40 °C until PCR amplification.

16S rRNA gene amplification and NGS sequencing. To characterize the bacterial communities present in the samples (see S1 File), the hypervariable V4 region of the 16S rRNA gene was targeted using the 515 F (GTGCCAGCMGCCGCGGTAA) and 806 R (GGGACTACHVGGGTWTCTAAT) primers[41]. The 16S rRNA

sequencing libraries were constructed according to the Illumina's 16S rRNA metagenomics sequencing library preparation protocol (Illumina, San Diego, CA, USA). During library preparation, the amplicon products were cleaned following each PCR round using magnetic beads from the AxyPrep Mag PCR Clean-up kit (AXYGEN, Big Flats, NY, USA), and the size of the amplicons was verified each time on a 1.5% agarose gel. One endogenous sample failed to generate PCR amplicons and was excluded. Illumina sequencing libraries were constructed for all 23 remaining samples (11 endogenous and 12 exogenous), and amplicon DNA concentration was measured with Quant-iT PicoGreen (Molecular Probes, Inc. Eugene, OR, USA). Final libraries were pooled in equimolar amounts. Illumina sequencing (250-bp paired-ends) was performed at the Microbiome Core Facility, School of Medicine, University of North Carolina, Chapel Hill, NC, USA.

Bioinformatics and statistical analyses. The sequencing data were processed using the Quantitative Insights Into Microbial Ecology 2 (QIIME2) program[42]. Illumina paired-end sequence reads were first demultiplexed to assign the sequences to their sample of origin using the QIIME2 demux emp-paired command and then quality filtered using the DADA2[43] algorithm as a QIIME2 plugin. Through the DADA2 pipeline, the paired-end reads were joined together, denoised, and chimeras, as well as residual PhiX reads, were removed. This results in the identification of all unique bacterial sequences called amplicon sequence variants (ASVs) which are technically equivalent to 100% OTUs but without errors[44,45]. Primers sequences were trimmed off the reads, but the sequences were not truncated to increase the chances of joining the paired-end reads efficiently. The 16S rRNA gene sequence variants (representative sequences) were assigned taxonomic classification using a pretrained Naïve Bayes classifier which was trained on the Greengenes database (gg_13_8, 99% OTUs, as recommended in QIIME2)[46]. Taxonomy was assigned using the Qiime feature-classifier classify-sklearn command. To calculate the statistical difference between the exogenous and endogenous groups, relative abundances of taxa with abundance higher than 1% (across all samples) were arcsine square root transformed, and a Welch two-sample *t*-test[47] was run using abundances from each sample as replicates. Relative abundance data were imported from QIIME2 into the R statistical software[48] for running the *t*-tests.

Prior to computing diversity metrics, the representative sequences obtained after DADA2 were aligned and masked to remove gaps, and a mid-point rooted phylogenetic tree was constructed using the QIIME2 FastTree 2 plugin[49]. To avoid biases due to sample-based variations in library sizes and to retain all of our samples in the subsequent analyses, each sample was rarefied to a depth of 18,000 sequences per sample. Alpha diversity was estimated by the Shannon index (integrates both richness and evenness)[50], the number of observed OTUs (ASVs), and Faith's phylogenetic index[51]. The statistical significance of alpha diversity between groups (exogenous and endogenous samples) was inferred using pairwise Kruskal-Wallis H-tests. Beta diversity was calculated using the weighted UniFrac[52] metric, and a PERMANOVA test was run to determine if there was any statistical difference between the two treatment groups. The principal coordinate analysis (PCoA) result was visualized using EMPEROR[53].

16S rRNA gene cloning and sequencing. The short-read length of sequences produced by the Illumina MiSeq platform limited taxonomic resolution during taxonomy assignment of the 16S rRNA gene sequences. To improve classification, especially for the most abundant taxa (relative abundance $\geq$10% across all samples), a nearly full-length fragment of the 16S rRNA gene was amplified using the universal bacterial primers, 27 f and 1492r[54] following a protocol previously described in Ponnusamy *et al.*[55]. DNA from four fly samples that contained the highest abundances of the targeted taxa were used (Table 1). In order to construct the 16S clone libraries, the PCR products were verified for their sizes on a 1.5% agarose gel stained with ethidium bromide and purified using the AxyPrep Mag PCR Clean-up kit. Purified PCR products from each sample were cloned into the pGEM-T Vector (Promega) as per manufacturer's instructions. Colonies were picked at random, and the presence of the insert was verified by amplifying the clones with the vector primers M13F (CCCAGTCACGACGTTGTAA AACG) and M13R (AGCGATAACAATTTCACACAGG). In total, 30 colonies with inserts were first Sanger sequenced using only the M13F primer, and the sequencing results were blasted against the BLASTn (Basic Local Alignment Search Tool) sequence database to identify homologous sequences and examine phylogenetic relationships. Then, four colonies (one per sample/targeted taxa) were further Sanger sequenced using two additional internal primers (520F, 968F or 518R, 984R) based on the direction in which the insert was cloned into the vector. Sanger sequencing for all samples was done at Eton Bioscience, Inc (Research Triangle Park, NC, USA).

Phylogenetic analyses of 16S rRNA gene clones. For each of the four colonies, each targeting a different taxon, the DNA sequences obtained using the M13F and two internal 16S rRNA gene primers were imported into the Molecular Evolutionary Genetics Analysis (MEGA version 7.0) software[56]. The sequences were aligned using the ClustalW program. Due to sequencing errors and low-quality reads towards the ends of the reads when using Sanger sequencing, a less error-bearing 16S rRNA gene DNA sequence was reconstructed by joining three DNA contigs (500-600 bp each) together using a minimum 100 bp overlap between the sequences. First, 16S rRNA gene sequences were screened for vector contamination using the NCBI VecScreen program and sequences that were identified to be of vector origin were removed. The final version of each 16S rRNA gene clone sequence was then blasted against the NCBI GenBank database using BLASTn. The 16S rRNA gene sequences were blasted against the NCBI 16S ribosomal RNA database on February 6, 2019. Clones with DNA sequences sharing 99-100% query coverage and at least 99% identity with GenBank sequences were assigned to that specific phylotype. Multiple alignments were performed using the ClustalW algorithm. The phylogenetic relationship between these clones and a selection of other closely related species was reconstructed using the kimura two-parameter model[57] and neighbour-joining algorithms as implemented in the MEGA7 software package.

Sample IDs	Porphyromonadaceae	Xanthomonadaceae	Moraxellaceae	Enterococcaceae	Enterobacteriaceae	Pseudomonadaceae	Flavobacteriaceae
Fi2	73.56	18.04	0	0.48	0.305	0.02	0
Fi3	47.51	27.5	0	1.6	0.101	0.03	0
Fi4	51.41	26.69	0	1.62	0.038	0.04	0
Fi5	6.18	12.19	0	5.27	0.25	0.046	0.05
Fi6	65.01	19.89	0	0.6	0.379	0.04	0
Fi7	89.87	5.8	0	1.17	0	0.02	0
Fi10	46.34	27.9	0	1.78	0.061	0.03	0
Fi11	72.36	17.38	0	0.96	0.151	0	0
Fi12	92.26	4.87	0	0.58	0	0	0
Fi13	7.18	13.17	0	5.1	0.34	0.07	0.11
Fi14	91.73	5.04	0	0.77	0.032	0.012	0
Fo1	34.33	23.85	21.18	3.53	2.15	1.3	2.11
Fo2	6.99	45.27	12.04	6.96	8.85	4.2	1.15
Fo3	7.69	43.36	11.86	9.44	7.6	4.2	1.12
Fo5	32.05	24.72	20.37	3.58	2.46	1.6	2.53
Fo6	17.59	25.99	20.92	6.85	1.54	1.4	4.7
Fo7	32.68	23.65	21.44	3.19	2.26	1.4	2.51
Fo10	7.36	43.53	11.81	8.41	8.1	4.1	1.1
Fo11	17.14	25.92	20.91	6.5	1.7	1.6	4.9
Fo12	12.8	18.47	36.04	6.55	2.86	3	3.11
Fo13	14.46	19.7	32.72	6.26	2.86	3.65	3.05
Fo14	12.04	17.86	34.95	7.92	2.78	4.9	2.65
Fo15	8.49	16.83	12.69	9.14	13.34	10.6	4.12
Average all	36.83	22.07	11.17	4.27	2.53	1.83	1.44

Table 1. Overall relative abundances (%) across samples at the family level. Sample IDs: Fi2 to Fi14, internal body (endogenous) sample; Fo1 to Fo15 external body (exogenous) sample.

Results

Data summary. DNA extraction from pooled washes (from surface sterilization) yielded no bands on agarose gel after PCR amplification and thus was not sequenced. A total of 23 16S rRNA gene libraries from exogenous and endogenous *P. regina* extracts for each fly separately were subjected to Illumina paired-end sequencing. A total of 10,622,697 16S rRNA gene sequences were obtained. After quality filtering using DADA2, 7,684,848 reads remained with an average of 334,124 reads (minimum of 18,504 and maximum of 517,162). The remaining sequences were clustered into 2,452 sequence variants and assigned to 27 phyla, 171 families and 533 genera (with some further classified into species).

Alpha and beta diversity measures. The asymptotic shape of the rarefaction curves of the observed OTUs (ASVs) suggests that our sequencing depth of 18,000 was sufficient to capture the majority of taxa present in the samples (Fig. 1). Comparisons between outside and inside samples of the black blow flies revealed significant differences in alpha diversities. In fact, the number of observed ASVs was significantly higher from the outside samples compared to inside samples (Kruskal-Wallis, $P < 0.001$) (Fig. 2A). Similarly, Shannon diversity (Kruskal-Wallis, $P < 0.001$) (Fig. 2B) as well as phylogenetic diversity (Kruskal-Wallis, $P < 0.001$) (Fig. 2C) were significantly higher outside.

Bacterial community difference between the two groups (β-diversity of exogenous vs. endogenous communities) was tested using the weighted Unifrac metric. This analysis revealed that the majority of the variation in bacterial diversity across the samples could be attributed to where the samples originated from (Fig. 3). Principal coordinates analysis showed distinct clustering of exogenous and endogenous samples, suggesting differences in the microbial communities of these groups (Fig. 3). This was further confirmed by a permutation multivariate analysis of variance (PERMANOVA) which found a significant difference between these groups at the β-diversity level (PERMANOVA, $P = 0.001$).

Bacterial community composition. Overall, the most sequence-abundant bacterial taxa at the phylum level were Proteobacteria (50.6%), Bacteroidetes (38.9%), Firmicutes (8.5%) and Actinobacteria (1.1%) (Fig. S2; see S2 File). Seven families were found to be the most sequence-rich and include Porphyromonadaceae (36.8%), Xanthomonadaceae (22.1%), Moraxellaceae (11.2%), Enterococcaceae (4.3%), Enterobacteriaceae (2.5%), Flavobacteriaceae (1.4%) and Pseudomonaceae (1.8%) (Table 1). At the highest taxonomic resolution (level 7, species level), most taxa were classified up to the genus level. In general, and across all samples, 6 genera were identified to be the most abundant: *Dysgonomonas* (36.8%), *Ignatzschineria* (20.6%), *Acinetobacter* (10.0%), *Vagococcus* (3.8%), *Myroides* (1.1%, further classified into species see Fig. 4) and Wohlfahrtiimonas (1.1%) (Fig. 4; see S2 File). These taxa were all differentially abundant between the two sites on the fly (Welch's t-test, $P < 0.05$). In the exogenous samples, *Dysgonomonas* (16.9%), *Ignatzschineria* (24.9%), *Acinetobacter* (19.2%), *Vagococcus*

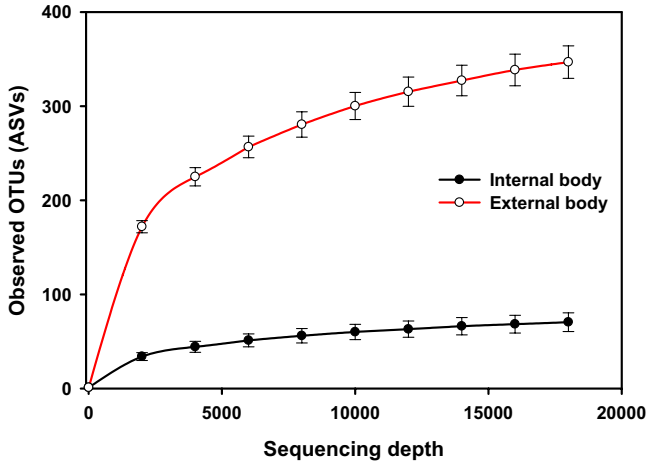


Figure 1. Rarefaction curves of the mean number of observed OTUs (sequence variants) in internal (endogenous) versus external body (exogenous) samples.

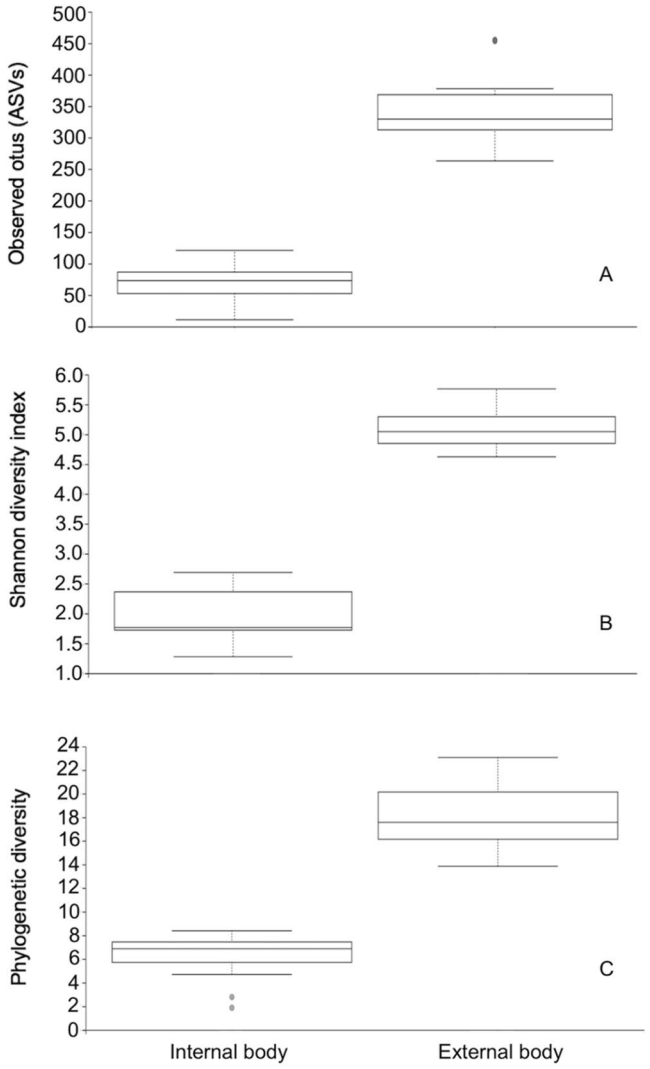


Figure 2. Alpha diversity measures of the internal (endogenous) and external body (exogenous) microbiomes of adult black blow flies. (**A**) Observed OTUs, (**B**) Shannon diversity and (**C**) Faith's phylogenetic diversity.

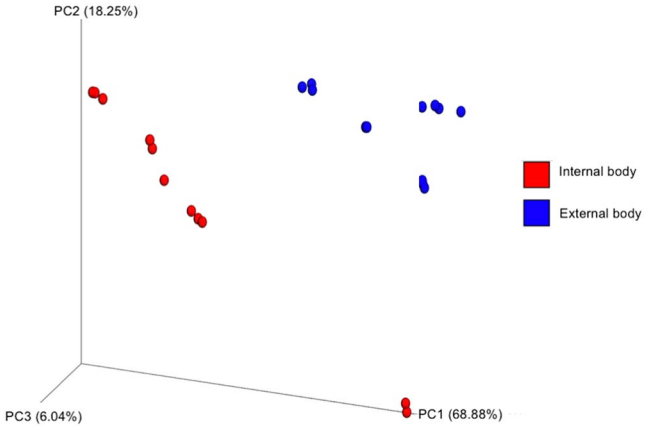


Figure 3. Principal coordinate analysis of bacterial composition between internal (endogenous) and external body (exogenous) samples of adult black blow flies. Analysis was based on the weighted Unifrac metric.

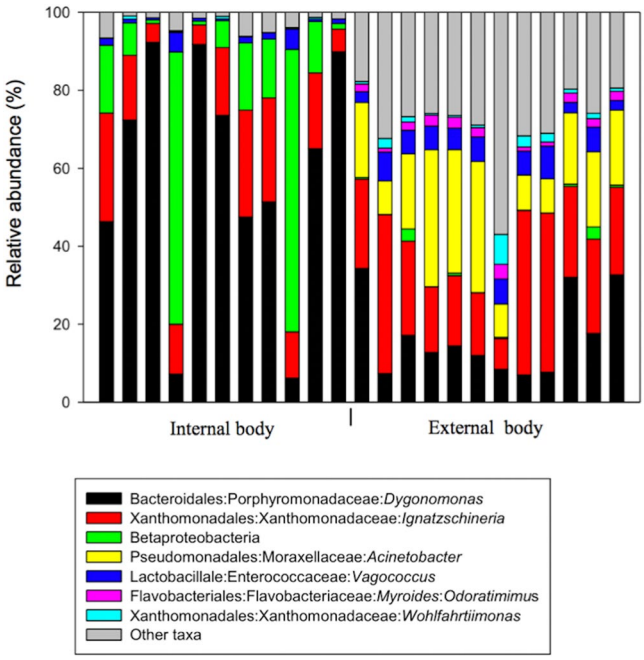


Figure 4. Relative abundance of major bacteria in internal (11 samples) and external body (12 samples) DNA samples from *P. regina* adults at the highest taxonomic resolution. Bars represent proportions of each taxa. "Other taxa" refers to all the taxa with relative abundance below 1% over the total number of reads. Ten per cent of the reads (10%) of the reads across all samples were classified as Betaproteobacteria. In the legend from left to right: Order:Family:Genus:Species.

(5.6%), *Myroides* (2.2%) and *Wohlfahrtiimonas* (1.8%) were the most abundant. The endogenous samples harbored *Dysgonomonas* (58.5%), *Ignatzschineria* (15.9%) and *Vagococcus* (1.8%), as the most abundant genera. *Myroides* (0.01%) and *Acinetobacter* (0.01%) were almost absent from the inside samples while *Wohlfahrtiimonas* had a very marginal relative abundance (0.3%) (Fig. 4). A bacterial taxon with 10% of the reads was classified only to the class level as a Betaproteobacteria (Fig. 4).

We also compared the bacterial diversity between individuals for the 7 predominant taxa found on the outside of the flies, the relative abundance of *Dysgonomonas* ranged from 7 to 34%; for *Ignatzschineria* ranged from 8 to 42%; for Betaproteobacteria ranged from 0.02 to 0.3%; for *Acinetobacter*, from 8 to 35%; for *Vagococcus*, from 2 to 8%; for *Myroides odoratimimus*, from 1 to 4%; for *Wohlfahrtiimonas*, from 0.4 to 8%. Furthermore, we detected several low relative abundance (<1%) bacteria (i.e. *Sphingobacterium multivorum*, *Faecalibacterium prausnitzii*, *Psychrobacter pulmonis*) found on some flies not found on others (see S2 File).

16S rRNA gene cloning and phylogeny results. All 30 16S rRNA cloned colonies that were Sanger sequenced using the M13 primers matched to their respective targeted taxa when the clone sequences were blasted against the NCBI database (http://blast.ncbi.nlm.nih.gov/). The nearly full-length reconstructed 16S rRNA gene

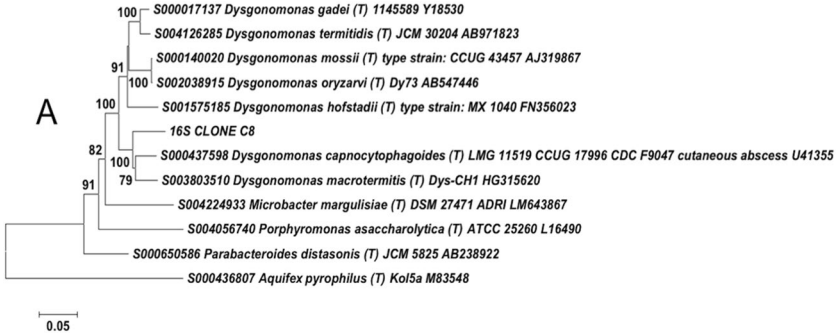


Figure 5. Neighbour-joining tree based on 16S rRNA gene sequences showing the relationship between cloned sequences from *Phormia regina* adults and sequences of other closely related bacterial species. Clone "16S CLONE C8" originated from sample (Fi12) that contained 92% of sequences assigned to the genus *Dysgonomonas*. *Aquifex pyrophilus* was used as an outgroup. The sequences were aligned using the ClustalW algorithm. Bootstrap values based on 500 replications, are given at the branching nodes. Bar represents 0.05 substitutions per nucleotide position.

clones had an average length of 1496 bp. The 16S rRNA gene clone sequence "Clone A1" matched to the species *Ignatzschineria ureiclastica* strain FFA (NR_116121.1) and *Ignatzschineria larvae* DSM 13226 (NR_025381.1) with 99% similarity. BLASTn searches with "Clone B9" affiliation to the species *Acinetobacter radioresistens* with 99% similarity to both the strains NBRC 102413 (NR_114070.1) and FO-1 (NR_026210.1). The first three highest BLAST matches for "Clone C8" were 16S rRNA gene sequences from *Dysgonomonas capnocytophagoides* strain JCM 16697 (NR_113133.1) (93%), *Dysgonomonas capnocytophagoides* strain CDC F9047 (NR_044778.1) (93%) and *Dysgonomonas alginatilytica* strain HUA-2 (NR_137388.1) (92%). The first four top hits after BLAST searches for "Clone D17" were: *Paraburkholderia rhizoxinica* strain HKI 454 (NR_102769.1) (94%), *Paraburkholderia rhizoxinica* strain HKI 454 (NR_042393.1) (94%), *Burkholderia rinojensis* strain A396 (NR_118637.1) (94%) and *Paraburkholderia endofungorum* strain HKI 454 (NR_042584.1) (94%).

The sequences used for constructing the trees were type species retrieved from the Ribosomal Database Project hierarchy browser (RDP; http://rdp.cme.msu.edu/)[58]. The 16S "CLONE A1" was placed within the genus *Ignatzschineria* (bootstrap value = 100) and more similar to *Ignatzschineria larvae* (Fig. S3). Clone B9 was more closely related to *Acinetobacter radioresistens* (bootstrap value = 100) within the *Acinetobacter* genus (Fig. S4). The placement of Clone C8 within the genus *Dysgonomonas* was well supported (bootstrap value = 100) and was more closely related to both *Dysgonomonas capnocytophagoides* and *Dysgonomonas macrotermitis* (Fig. 5). The phylogenetic tree revealed that the cloned sequence "Clone D17" was placed in a completely different clade from the genus *Burkholderia sensus lato* (Fig. S5).

Discussion

In this study, we investigated, for the first time, the bacterial microbiome associated with the internal body (endogenous) and external body surfaces (exogenous) of *Phormia regina* adults collected from the wild in a suburban area but associated with a multi-use animal and Ag research production system in the NCSU campus. The study revealed the presence of several bacterial taxa that were, most of the time differentially abundant between the two environments. The results also showed that these flies harbored an appreciable number of potentially pathogenic as well as several potentially multi-drug resistant bacteria.

Our findings revealed the number of observed ASVs (OTUs), Shannon diversity, and Faith's phylogenetic diversity were all significantly higher in the outside samples representing DNA extracted from the surface of each fly. This demonstrates that diversity was higher on the external surface of the fly's body compared to the gut and therefore the flies harbored more different bacterial species on the outside of their bodies. This result was not unexpected and has been shown previously in flies. For example, using culture-dependent techniques, Adeyemi *et al.*[59] demonstrated that both the number and variety of bacteria isolated from external surfaces of filth flies were significantly higher compared to the gut. One possible reason for higher diversity on the fly surface is the availability of high microbe-laden feeding/breeding sites; black blow flies are known for example to frequently visit human and/or animal dung[60]. In addition, blow flies, house flies, and flesh flies have adhesive pads between the tarsal claws of their legs called pulvilli which are covered in fine hairs or "tenant setae". The setae have been shown to secrete an adhesive substance to increase surface area for attachment to smooth surfaces but are also deemed to retain many microorganisms on the legs[21,61–63]. Microorganisms also attach to other body parts such as the wings, the mouthparts and abdomen[21,62].

Although the transfer of bacteria between flies during the trapping period cannot be ruled out, there was no indication that the trapping method was an issue. Alpha diversity and bacterial community composition analyses showed that the exogenous microbiomes were not homogenous between the flies; variability was determined to have resulted from differences in bacterial composition and taxonomic abundances between samples. Additionally, minor taxa (abundances <1%) included several bacterial species that were detected on some flies while absent (relative abundance of 0%) on others, suggesting that the exogenous samples were independent. Further, the timing (November) and the methods by which trapping was conducted permitted the collection of a

single species (*Phormia regina*). Trapping of a single species mitigated potential contamination of our target flies by bacteria associated with other flies.

As shown in the PCoA plot for the abundance-weighted UniFrac distance, the exogenous samples formed a cluster that is separated from the endogenous samples. This could be due to the differential abundance of some important bacterial taxa between the two environments but also to the presence/absence aspects of those taxa from one environment to another (discussed more later).

Overall, Proteobacteria, Bacteroidetes, Firmicutes and Actinobacteria were the predominant phyla in our samples. This result is in agreement with findings in houseflies[64], fruit flies[65], flesh flies[66] and other blow flies[21,37,67]. It is worth noting that the overall average relative abundances of the last four families were essentially driven by outside samples as members from those families were barely detected in inside samples (abundances ranging between 0 and 0.15%). No Moraxellaceae was detected inside. A similar trend was also observed by Weatherbee *et al.*[37] who investigated the internal microbial communities of third-instar specimens of *P. regina* as well as the external microbiome of the larval mass (larva aggregate composed of multiple fly species including *P. regina*) present on swine carcasses at different time points. At 132 and 156 h after carcass placement, where *P. regina* larvae were the most predominant species (over 85% of the larval mass), several taxa at the family level identified outside were not detected within the larvae. These results suggest that both the immature and adult stages of *P. regina* may selectively control the gut bacteria either through digestion or other unknown mechanisms. Several families (Bacteroidaceae and Bifidobacteriaceae) identified among the 10 most abundant families in the swine carcass study were marginally present in our samples while Porphyromonadaceae, the most abundant family taxon found internally in this study, was not present and/or abundant within *P. regina* larvae. These differences may be due to several factors including developmental stage-related differences (adults and larvae may be differentially associated with certain types of bacteria), the geographic locations of the studies (Indiana vs. North Carolina) as well as the feeding sources available to the flies and different bacteria or differences in bacteria abundance in the available feeding sources. All those factors have been shown to significantly impact the microbiomes of several insects[68].

At the genus level, *Dysgonomonas*, *Ignatzschineria*, *Acinetobacter*, *Vagococcus*, *Myroides*, and *Wohlfahrtiimonas* were the most predominant taxa in our study. These genera, even though their relative abundance may vary, have been isolated from various insect species before. The majority of them, however, are frequently detected together (in 16S-based NGS studies) and often times in high abundances in insects associated with filth and most particularly in saprophagous and/or necrophagous insects/flies[64–67,69,70]. This result may be due to similar ecological niches in those insects and their overlapping range of feeding sources, an adaptation of these bacterial taxa to physicochemical conditions of the guts of these insects and to the role that these microorganisms may be playing in host nutrition and/or fitness.

Dysgonomonas, the genus with the highest abundance has been isolated from a burying beetle *Nicrophorus vespilloides* (Herbst) (Coleoptera: Silphidae)[69], a bombardier beetle *Brachinus elongatus* (Chaudoir) (Coleoptera: Carabidae)[45], a wood-feeding termite *Reticulitermes speratus* (Kolbe) (Isoptera: Rhinotermitidae)[71], the common house fly *Musca domestica* (Diptera: Muscidae)[64], the oriental latrine fly *Chrysomya megacephala* (Diptera: Calliphoridae)[67], the Australian sheep blow fly, *Lucilia cuprina* (Wiedmann) and the common green bottle fly *Lucilia sericata* (Meigen) (Diptera: Calliphoridae). Similarly, results from analyses of this genus isolated from termites, dung beetles, black soldier flies and mosquitoes suggest that *Dysgonomonas* is implicated in the breakdown of carbohydrates namely cellulose and hemicellulose as well as the production of vitamin B12[72–76]. Whether *Dysgonomonas* plays similar roles in the black blow fly is yet to be confirmed. However, the high abundance of this genus in internal samples in this study points to that direction.

Ignatzschineria[77] and *Wohlfahrtiimonas*[78] were both originally described in the larvae of a parasitic fly, the spotted flesh fly *Wohlfahrtia maginifica* (Schiner) (Diptera: Sarcophagidae) a major myasis agent in animals[66]. Phylogenetically, they are two related genera but belong to two different lineages. These two genera have been isolated from other flesh flies[66,79], house flies[64], blow flies[67,70] and carrion beetles[69]. Little is known about the role that members of these genera play in insects. However, they both exhibit strong chitinase activity, and Toth *et al.*[77] have suggested a possible role of *Ignatzschineria* in metamorphosis. The role that they play in adult insects needs investigation. The genus *Vagococcus* was previously isolated from fruit flies[80], house flies[64], blow flies[21,70], red imported fire ants, *Solenopsis invicta*[81] and burying beetles[69]. They are deemed to produce antiviral metabolites in *Aedes albopictus*[82], but nothing is known about their role in *Phormia regina*.

Acinetobacter was almost exclusively located externally (abundance inside was 0.01%). In fact, *Acinetobacter* spp. are widespread in natural environments and thus are frequently recovered from soil, polluted water, plants, animals and humans[83]. However, they have also been isolated from the guts of different insects[84,85]. For example, Coon *et al.*[86], showed that the inoculation of *Acinetobacter* spp. restored larval development in axenic mosquito larvae while Minard *et al.*[87] suggested they aided in the digestion of plant nectar consumed by *Aedes albopictus* mosquitoes. It was also suggested that they may help detoxify defensive plant compounds for tropical herbivorous beetles[88]. They also secrete antiparasitic compounds in the guts of several insects[89]. The presence of this genus on the outer body of the flies is probably of environmental origin; they may have been picked up mechanically from a contaminated source and may not have a function role in fly biology. It would be interesting to investigate if the bacteria could sustain the black blow fly gut environment and what possible role those bacteria could play.

Myroides odoratimimus, previously known as *Flavobacterium odoratum*[90], was also exclusively observed in outside samples (abundance inside was 0.01%). Like some of the previous taxa described in this section, *M. odoratimimus* are ubiquitous in natural environments and are commonly found in soil, fresh and marine water, meat-processing plants and insects[21,90,91]. *Myroides* spp. are well known for their ability to secrete antimicrobial substances[90], and thus it is possible that they help protect insects that harbor them against other pathogenic bacteria.

Based on our findings in *P. regina*, the absence of some taxa on the outside of the fly that are found internally and the fact that the most abundant taxa found inside are also present on the fly's surface suggests that the microbial communities associated with black blow flies are acquired from the environment probably from feeding/breeding sites. Also, based on previous fly research, it is likely that the internal bacteria present may contribute to the host's development. Most of the bacterial taxa identified in the *P. regina* microbiome contained known pathogens (discussed in more detail below).

Most reads (89%) obtained from Illumina sequencing were classified down to the genus level even for the most sequence-rich taxa (except for Betaproteobacteria and *Myroides odoratimimus*) thus limiting the depth of analysis of the results. A literature search revealed that some of those genera are well known to be pathogenic microorganisms. To improve taxonomic resolution and to verify the detection of potential pathogens in our samples, the first four identified taxa in terms of relative abundance (≥10%, *Dysgonomonas*, *Ignatzschineria*, *Betaproteobacteria and Acinetobacter*) were further targeted by developing nearly full length 16S rRNA gene clones and sequencing them using Sanger sequencing. The results showed some of the most abundant taxa in our samples may be new bacterial taxa and also confirmed the existence of potential human, animal and plant pathogens in our fly samples.

Our results suggest that some of our samples contained a new genus in Burkholderiaceae: Ten percent of the reads was classified as Betaproteobacteria, a class in the phylum Proteobacteria. BLASTn searches for "Clone D17" which was targeting that class for better identification revealed that the clone sequence was 94% similar to two strains of *Paraburkholderia rhizoxinica*, a strain of *Burkholderia rinojensis* and a strain of *Paraburkholderia endofungorum*. During phylogenetic analyses, Clone D17 was placed in a completely different clade from the genus *Burkholderia sensus lato* (s.l.). Therefore, the bacterium corresponding to that sequence constitutes a distinct lineage with no closely related allied 16S rRNA gene sequences and probably represents a novel genus in the family Burkholderiaceae, order Burkholderiales, class Betaproteobacteria in the phylum Proteobacteria. The genus *Burkholderia sensus lato* (s.l.) is the original genus described by Yabuuchi *et al.*[92]. Since then, the taxonomy of this genus has been evaluated several times with the addition of several new taxa, and today it is subdivided into 5 different groups or "sub-genera"[93,94]. The most interesting issue is that during the phylogenetic tree reconstruction, we made sure to include at least one member from each of the five groups (based on type species present in RDP), but Clone D17 was placed as a sister group to *Burkholderia* and *Pandoraea* with strong support (bootstrap value = 93). No assumptions can be made at the current stage about the pathogenicity of this potentially new genus.

Our results indicate that our samples contained a new *Dysgonomonas* species: The taxa previously classified as *Dysgonomonas* was targeted with Clone C8. The sequence from this clone had 93% similarity with two strains of *Dysgonomonas capnocytophagoides* and 92% similarity with a strain of *Dysgonomonas alginatilytica* based on BLASTn searches. Phylogenetically, it clustered with all the other *Dysgonomonas* (bootstrap value = 100) but was more closely related to both *Dysgonomonas capnocytophagoides* and *Dysgonomonas macrotermitis* with strong statistical support (bootstrap value = 100). These results suggest that this molecular isolate represents a potential new species within the genus *Dysgonomonas*, family Porphyromonadaceae, order Bacteroidales, class Bacteroidia in the phylum Bacteroidetes. *Dysgonomonas capnocytophagoides*, formerly known as CDC group DF-3 (Center for Disease Control group dysgonic fermenter-3) is an opportunistic bacterium associated with diarrhea and bacteremia[95,96]. *Dysgonomonas macrotermitis*, on the other hand, was only recently isolated from a fungus-growing termite[72,97] and has not been associated with any disease so far. More work is needed to better characterize the bacterium represented by Clone C8 (this study).

New *Ignatzschineria* species: The sequence from "Clone A1" matched to the species *Ignatzschineria ureiclastica* and *Ignatzschineria larvae* with 99% similarity based on BLASTn searches conducted in February 2019. Clone A1 clustered together with all the species in the genus *Ignatzschineria* (bootstrap value = 100) in the phylogenetic analyses but was more closely related to *Ignatzschineria larvae* (bootstrap value = 75) which can cause bacteremia[98]. This finding is indicative of a potential new species in the genus *Ignatzschineria*, family Xanthomonadaceae, order Xanthomonadales, class Gammaproteobacteria within the phylum Proteobacteria.

Acinetobacter radioresistens: Both BLASTn searches and phylogenetic analyses revealed the sequence of Clone B9 belonging to the bacterium, *A. radioresistens*. It was originally isolated from cotton and soil[99]. Like several other members of the genus *Acinetobacter*, this species has emerged as an opportunistic human pathogen. It can cause septicemia[100], nosocomial bloodstream infection and community-acquired infections in immune-compromised patients[101,102] and has the potential to damage human epithelial kidney cells through the production of outer membrane vesicles (OMVs)[103]. *A. radioresistens* have also been shown recently to cause nosocomial bacterial infection outbreaks in research animals[104]. Another concerning fact about this species is its ability to withstand harsh environmental conditions as well as its strong and well-established resistance to several antimicrobials. In fact, *A. radioresistens* is highly resistant to desiccation and radiation[105] and is known to be resistant to Carbapenem, a class of antibiotic normally highly effective against multidrug-resistant bacteria[102,103,106,107].

Myroides odoratimimus: This bacterium is also an opportunistic pathogen causing a variety of diseases/infections of which some are life-threatening. *M. odoratimimus* can cause bacteremia, endocarditis, pericarditis, pneumonia and septic shock, urinary tract infections, calcaneal ulcer and several other important bacteria-related infections in humans[64,66,108–111]. This species can also cause lethal infections in fish[112].

In addition to the taxa reported here as the most abundant (≥1%), it is important to note the detection in our samples of *Pseudomonas viridiflava* (relative abundance of 0.7%), a multi host plant pathogen. *P. viridiflava* causes foliar and stem necrosis as well as stem and root rot in a wide range of plants such as tomato, melon and eggplant[113–115]. Finally, in insects, *Wohlfahrtiimonas chitiniclastica*, the most frequently identified species of the genus *Wohlfahrtiimonas* is known to cause human infections[21,66].

In summary, this study provides, for the first time, a snapshot of the bacterial communities harbored both internally and externally by free-flying, black blow flies. The results revealed that the flies harbored a higher

diversity of bacterial species on their body surfaces probably owing to their ecology and behavior. Even though the majority of the most abundant bacterial taxa identified in this study have also been reported in other flies/insects, the presence of many potentially new taxa further confirms the necessity to conduct microbiome studies in insects in general but more importantly in synanthropic insects that breed in microbe-rich substrates such human and animal dung. Arguably, the most intriguing fact is the identification of known human, animal and plant pathogens such as *Myroides odoratimimus*, *Acinetobacter radioresistens* and *Pseudomonas viridiflava* on the fly body of which several are also resistant to several antibiotics.

Data availability

The next-generation sequencing data associated with this study have been deposited in GenBank under SRA accession: PRJNA554278. Cloned sequences have been deposited in GenBank under accession numbers: MN172461-MN172464.

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Author contributions

J.Z., A.C. and S.D. collected the fly samples, and J.Z. extracted DNA from the samples. J.M.D., J.Z. and N.T. prepared the libraries for next-generation sequencing. J.M.D., N.T. and L.P. analyzed the data. J.M.D. wrote the original draft of the manuscript, and J.M.D., N.T., J.Z., A.C., S.D., M.H.R., D.W.W., R.M.R. and L.P. reviewed and edited the final version of the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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(54) **METHODS AND COMPOSITIONS FOR KILLING OF INSECT PEST EGGS**

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(57) **ABSTRACT**

The invention is directed to ovicidal compositions comprising at least 2-undecanone and/or 2-tridecanone, and methods for the use of the compositions for the killing of insect pest eggs.

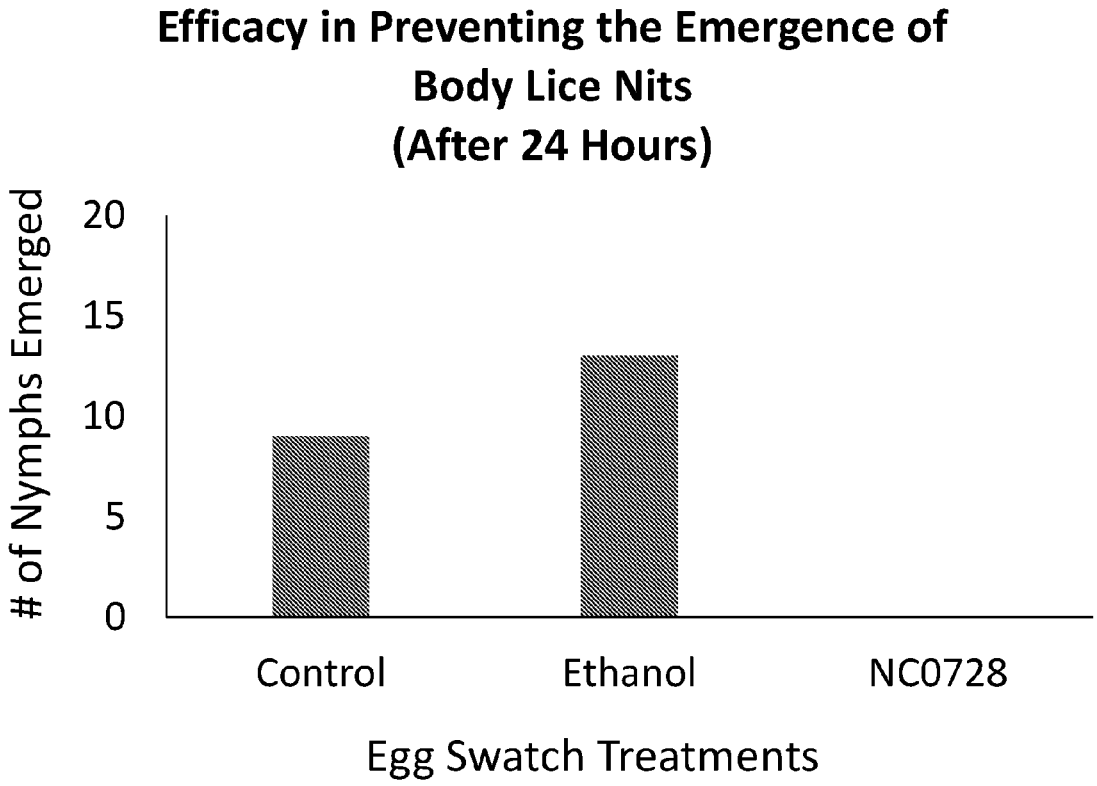


Fig. 1A

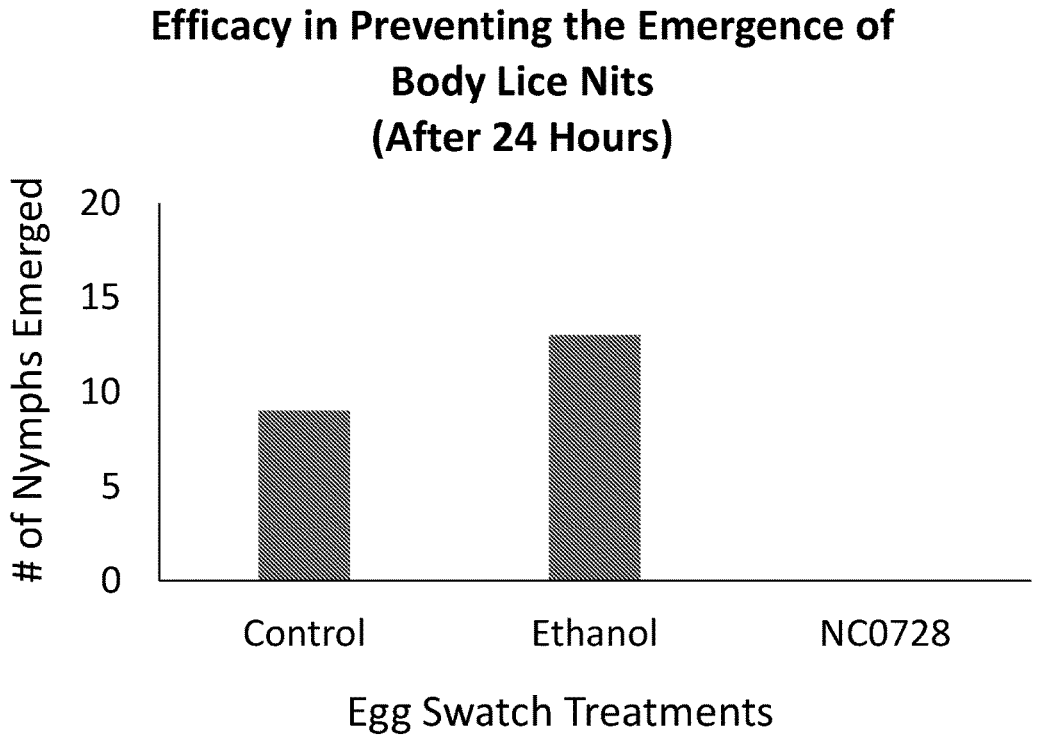

Efficacy in Preventing the Emergence of Body Lice Nits (After 24 Hours)
# of Nymphs Emerged
20
15
10
5
0
Control
Ethanol
NC0728
Egg Swatch Treatments

Fig. 1B

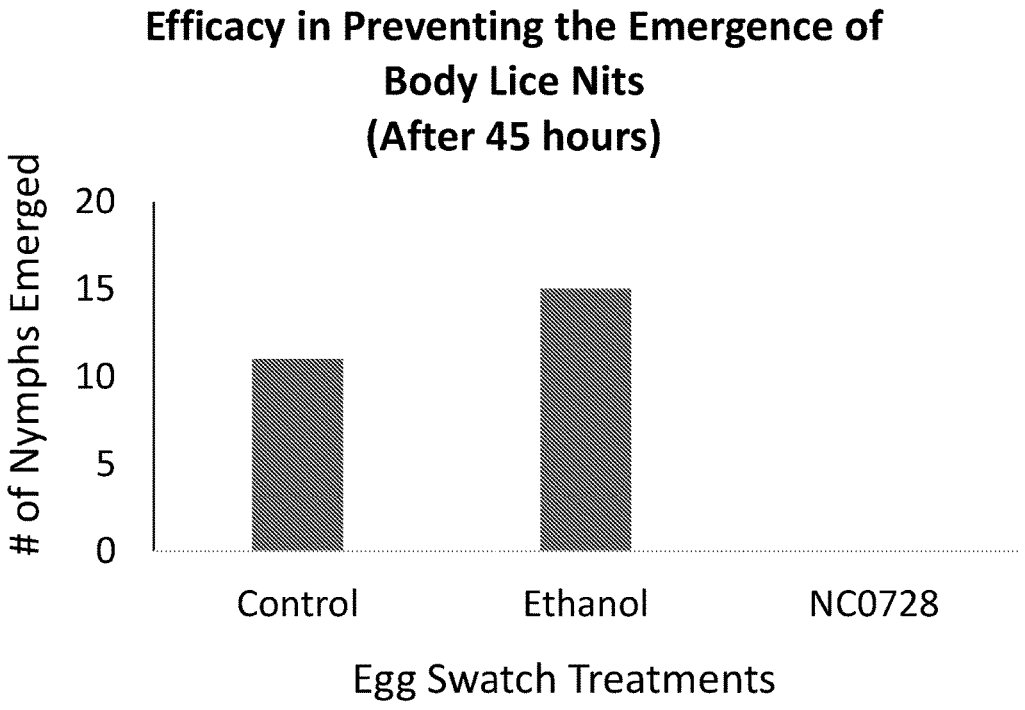

Efficacy in Preventing the Emergence of Body Lice Nits (After 45 hours)
# of Nymphs Emerged
20
15
10
5
0
Control
Ethanol
NC0728
Egg Swatch Treatments

Fig. 2

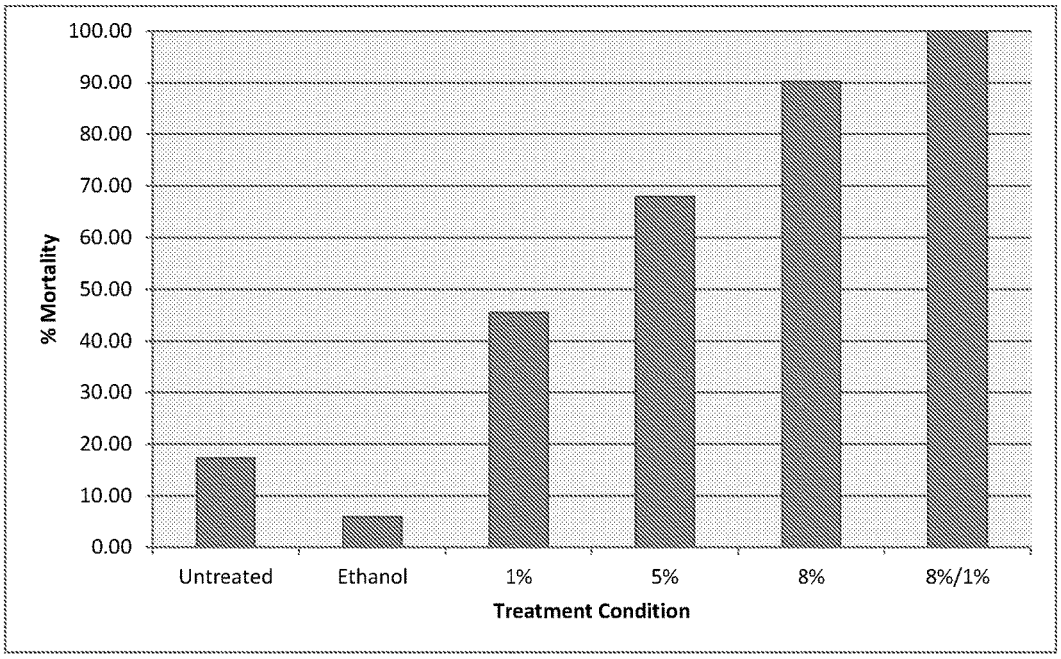

100.00
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
% Mortality
Untreated
Ethanol
1%
5%
8%
8%/1%
Treatment Condition

Fig. 3

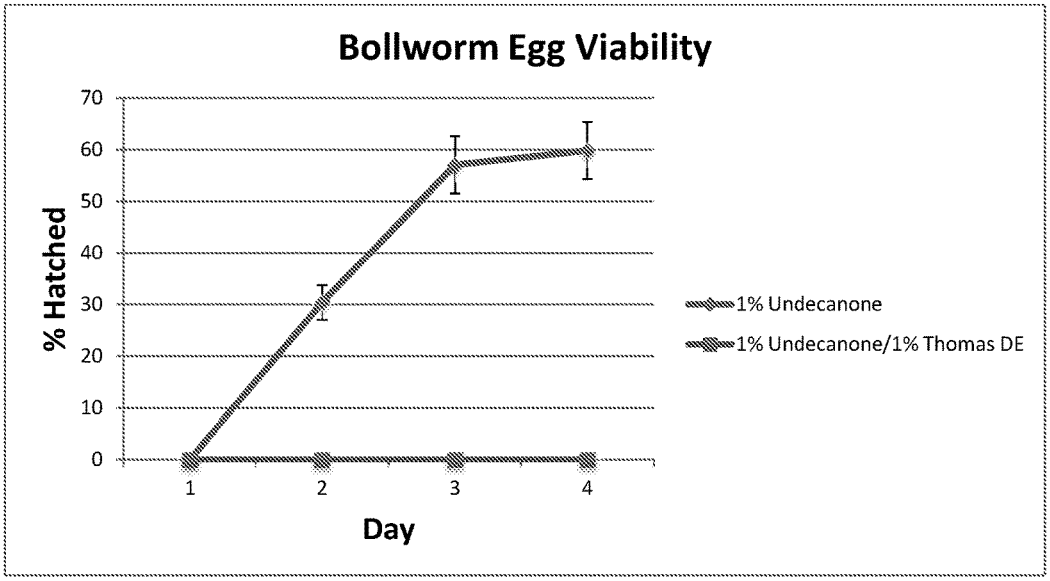
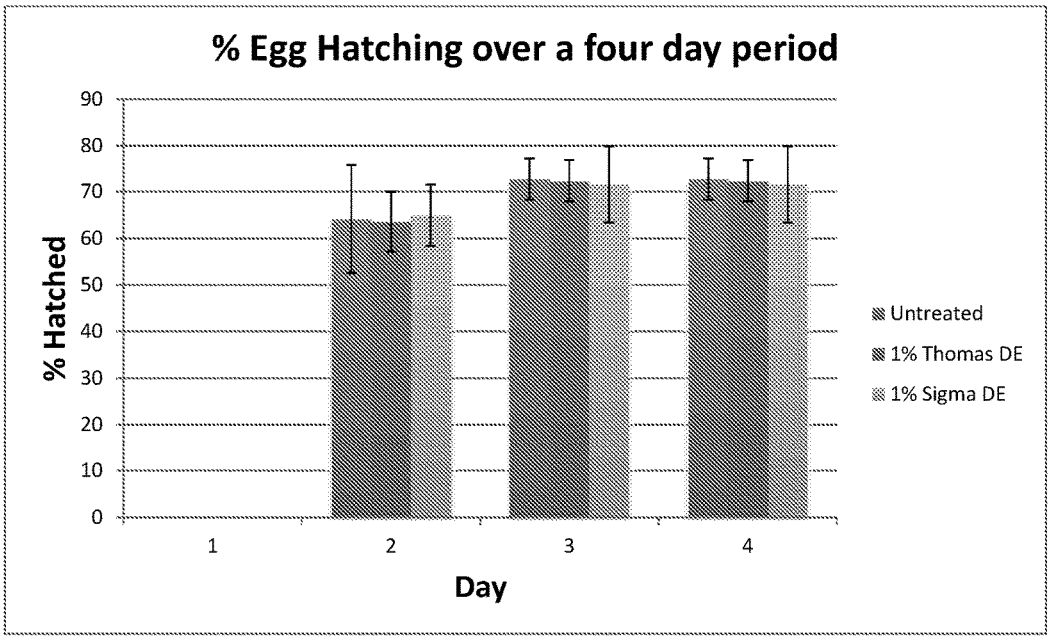

Bollworm Egg Viability
% Hatched
70
60
50
40
30
20
10
0
1
2
3
4
Day
1% Undecanone
1% Undecanone/1% Thomas DE

Fig. 4


% Egg Hatching over a four day period
% Hatched
90
80
70
60
50
40
30
20
10
0
1
2
3
4
Day
Untreated
1% Thomas DE
1% Sigma DE

METHODS AND COMPOSITIONS FOR KILLING OF INSECT PEST EGGS

STATEMENT OF PRIORITY

[0001] This application claims the benefit, under 35 U.S. C. § 119 (e), of U.S. Provisional Application No. 62/371,314; filed on Aug. 5, 2016 in the United States Patent and Trademark Office, the entire contents of which is incorporated by reference herein.

FIELD OF THE INVENTION

[0002] The present invention concerns compositions and methods for the control of insect pest eggs.

BACKGROUND OF THE INVENTION

[0003] The ability to control insect pests is an ongoing problem. In particular, even when a composition may be effective in killing insects, the eggs are typically not similarly destroyed. This allows for a cycle of re-infestation requiring repeated treatments. Further many insect pests are now resistant to the available pesticides. Thus, a need exists for new compositions and formulations that can control insect pests and their eggs.

SUMMARY OF THE INVENTION

[0004] One aspect of the invention provides a method of killing insect pest eggs, comprising contacting the insect eggs with an effective amount of a composition comprising 2-undecanone and/or 2-tridecanone, thereby killing the insect eggs.

[0005] A second aspect provides a method of killing lice eggs, comprising contacting the lice eggs with an effective amount of a composition comprising 2-undecanone and/or 2-tridecanone, thereby killing the lice eggs.

[0006] A third aspect provides a method of killing lice eggs on a subject infested with lice eggs or suspected of being infested with lice eggs, comprising applying to the subject an effective amount of a composition comprising 2-undecanone and/or 2-tridecanone, thereby killing the lice eggs on the subject.

[0007] A fourth aspect of the invention provides a method of killing insect eggs, comprising contacting the insect eggs with an effective amount of a composition comprising 2-undecanone and/or 2-tridecanone, and diatomaceous earth, thereby killing the insect eggs.

[0008] A fifth aspect provides a method of killing lice eggs, comprising contacting the lice eggs with an effective amount of a composition comprising 2-undecanone and/or 2-tridecanone, and diatomaceous earth, thereby killing the lice eggs.

[0009] A sixth aspect provides a method of killing lice eggs on a subject infested with lice eggs or suspected of being infested with lice eggs, comprising applying to a subject in need thereof an effective amount of a composition comprising 2-undecanone and/or 2-tridecanone, and diatomaceous earth, thereby killing the lice eggs on the subject.

[0010] These and other aspects of the invention are set forth in more detail in the description of the invention below.

BRIEF DESCRIPTION OF THE DRAWINGS

[0011] FIG. **1**A-**1**B shows the effect of 100% ethanol or a 15% 2-undecanone formulation (NC0728) on body lice eggs 24 hours (FIG. **1**A) and 45 hours (FIG. **1**B) after treatment.

[0012] FIG. **2** shows the percent mortality of bollworm eggs treated with 1%, 5%, 8% 2-undecanone or 8% 2-undecanone plus 1% DE (Thomas Labs).

[0013] FIG. **3** shows the bollworm egg viability following treatment with (1) 1% undecanone in ethanol (♦) or (2) 1% undecanone/1% DE (Thomas Labs) in ethanol (■).

[0014] FIG. **4** shows the percentage of bollworm eggs that hatched over a four day period following (1) no treatment or (2) treatment with two types of diatomaceous earth (DE), 1% Thomas Labs DE or (3) 1% Sigma DE.

DETAILED DESCRIPTION

[0015] The present invention now will be described hereinafter with reference to the accompanying drawings and examples, in which embodiments of the invention are shown. This description is not intended to be a detailed catalog of all the different ways in which the invention may be implemented, or all the features that may be added to the instant invention. For example, features illustrated with respect to one embodiment may be incorporated into other embodiments, and features illustrated with respect to a particular embodiment may be deleted from that embodiment. Thus, the invention contemplates that in some embodiments of the invention, any feature or combination of features set forth herein can be excluded or omitted. In addition, numerous variations and additions to the various embodiments suggested herein will be apparent to those skilled in the art in light of the instant disclosure, which do not depart from the instant invention. Hence, the following descriptions are intended to illustrate some particular embodiments of the invention, and not to exhaustively specify all permutations, combinations and variations thereof.

[0016] Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. The terminology used in the description of the invention herein is for the purpose of describing particular embodiments only and is not intended to be limiting of the invention.

[0017] All publications, patent applications, patents and other references cited herein are incorporated by reference in their entireties for the teachings relevant to the sentence and/or paragraph in which the reference is presented.

[0018] Unless the context indicates otherwise, it is specifically intended that the various features of the invention described herein can be used in any combination. Moreover, the present invention also contemplates that in some embodiments of the invention, any feature or combination of features set forth herein can be excluded or omitted. To illustrate, if the specification states that a composition comprises components A, B and C, it is specifically intended that any of A, B or C, or a combination thereof, can be omitted and disclaimed singularly or in any combination.

[0019] As used in the description of the invention and the appended claims, the singular forms "a," "an" and "the" are intended to include the plural forms as well, unless the context clearly indicates otherwise.

[0020] Also as used herein, "and/or" refers to and encompasses any and all possible combinations of one or more of the associated listed items, as well as the lack of combinations when interpreted in the alternative ("or").

[0021] The term "about," as used herein when referring to a measurable value such as a dosage or time period and the like refers to variations of ±20%, ±10%, ±5%, ±1%, ±0.5%, or even ±0.1% of the specified amount.

[0022] As used herein, phrases such as "between X and Y" and "between about X and Y" should be interpreted to include X and Y. As used herein, phrases such as "between about X and Y" mean "between about X and about Y" and phrases such as "from about X to Y" mean "from about X to about Y."

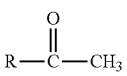
[0023] The term "comprise," "comprises" and "comprising" as used herein, specify the presence of the stated features, integers, steps, operations, elements, and/or components, but do not preclude the presence or addition of one or more other features, integers, steps, operations, elements, components, and/or groups thereof.

[0024] As used herein, the transitional phrase "consisting essentially of" means that the scope of a claim is to be interpreted to encompass the specified materials or steps recited in the claim and those that do not materially affect the basic and novel characteristic(s) of the claimed invention. Thus, the term "consisting essentially of" when used in a claim of this invention is not intended to be interpreted to be equivalent to "comprising."

[0025] As used herein, the terms "increase," "increasing," "increased," "enhance," "enhanced," "enhancing," and "enhancement" (and grammatical variations thereof) describe an elevation of at least about 25%, 50%, 75%, 100%, 150%, 200%, 300%, 400%, 500% or more as compared to a control.

[0026] As used herein, the terms "reduce," "reduced," "reducing," "reduction," "diminish," and "decrease" (and grammatical variations thereof), describe, for example, a decrease of at least about 5%, 10%, 15%, 20%, 25%, 35%, 50%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 100% as compared to a control. In particular embodiments, the reduction can result in no or essentially no (i.e., an insignificant amount, e.g., less than about 10% or even 5%) detectable activity or amount.

[0027] The present invention provides a method of killing an insect pest egg, comprising contacting the insect pest egg or applying to a subject or substrate, in an amount effective to kill the insect pest egg, a compound of Formula I:



(I)

[0028] wherein R is C_4-C_{20} linear or branched alkyl. In some embodiments, the compound of Formula I is linear. In some embodiments, the compound of Formula I may be C_7 to C_{13} alkyl. In representative embodiments, the compound comprises 2-undecanone (R is C_9) and/or 2-tridecanone (R is C_{11}). In particular embodiments, the insect pest is a louse.

[0029] As used herein, the term "alkyl" (e.g., alkyl, alkylcarboxy, alkylphenyl, etc.) refers to a straight or branched chain hydrocarbon having from 4 to 20 carbon atoms. The alkyl may optionally be substituted with substituents selected from the group which includes halo, lower alkoxy, lower alkylsulfanyl, lower alkylsulfenyl, lower alkylsulfonyl, oxo, hydroxy, mercapto, amino optionally substituted by alkyl, carboxy, carbamoyl optionally substituted by alkyl, aminosulfonyl optionally substituted by a substituent selected from the group including alkyl, nitro, cyano, halo and lower perfluoroalkyl, multiple degrees of substitution being allowed. Examples of "alkyl" as used herein include, but are not limited to, n-butyl, n-pentyl, isobutyl, pentyl, hexyl, octyl, nonyl, undecyl, the like.

[0030] The term "lower" as used herein with respect to alkyl moieties means linear or branched chain hydroxycarbon having from 1-4 carbon atoms

[0031] Subjects to be treated with compounds of the present invention include both human and animal subjects (e.g., dogs, cats, horses, cattle). Subjects may be directly or indirectly treated, such as by applying the active compound to the skin of the subject, or by applying the active compound to an article worn by or otherwise protecting the subject.

[0032] In some embodiments, substrates may be treated with compounds of the present invention. Such substrates include, but are not limited to, fabrics (e.g., clothing, towels, bed clothing, and the like), floors, plants, containers, walls, pools or open bodies of water, air, soil, nets, paper, trash containers, dumps, etc.

[0033] The compounds of the invention may be present in a composition, e.g., a formulation suitable for delivery of the compounds. The compositions (2-undecanone and/or 2-tridecanone) as described herein can be formulated in a variety of ways, including but not limited to mixing with a solid carrier, a liquid carrier, or a gaseous carrier. In addition, a surfactant, a sticking agent, a dispersion agent, a stabilizer, and other auxiliaries or additives may be added.

[0034] A formulation for the present invention includes but is not limited to an oil solution (oil emulsion), an emulsifiable concentrate, a wettable powder, a flowable formulation, granules, dusts, an aerosol (e.g., powdered, liquid), a shampoo, a conditioner, a creme rinse, a mousse, a foam, a lotion, a gel, a powder (e.g., a dry shampoo), a paste, or an ointment.

[0035] A solid carrier useful for the formulation may include, but is not limited to, fine powder or granules of cellulose, clays (e.g. kaolin clay, diatomaceous earth, synthetic hydrated silicon oxide, bentonite, Fubasami clay, acid clay), talc, ceramics, and/or other inorganic minerals (e.g. sericite, quartz, sulfur, active carbon, calcium carbonate, hydrated silicon oxide). In representative embodiments, the formulation comprises 2-undecanone and/or 2-tridecanone, and diatomaceous earth. Diatomaceous earth (DE) is formed from the fossilized remains of diatoms, the skeletons of which contain silica. The particle size of DE is typically about 1 μm to about 200 μm and the chemical composition is about 80 to 90% silica, with about 2 to 4% alumina and about 0.5 to 2% iron oxide.

[0036] Liquid formulations may be aqueous-based or non-aqueous (i.e., organic solvents), or combinations thereof, and may be employed as a foam, a gel, a suspension, an emulsion, a microemulsion, an emulsifiable concentrate, a shampoo, a conditioner, a creme rinse, a mousse, a lotion, a paste, an ointment, and the like. The ingredients may include rheological agents, surfactants, emulsifiers, dispersants, or polymers. Thus, a liquid carrier useful for the formulation may include, but is not limited to, water; organic solvents

such as alcohols including, but not limited to, methanol and ethanol; or vegetable/plant oils including but not limited to soybean oil and/or cottonseed oil.

[0037] Thus, in representative embodiments, a composition or formulation of the invention may be contacted with or applied to a subject in the form of a dry shampoo, liquid shampoo, conditioner, creme rinse, mousse, foam, lotion, ointment, and/or aerosol that comprises the composition. In some embodiments, the compositions or formulations may be provided in a microencapsulated form. In representative embodiments, a formulation may be a dry shampoo, which may be applied in any manner, for example, as a spray or as a dry powder. For example, a spray may comprise 2-undecanone or 2-tridecanone, and DE and an alcohol applied using an aerosol spray can. Alternatively, the dry shampoo may comprise 2-undecanone or 2-tridecanone, and DE that may be applied using a squeeze bottle.

[0038] An example gaseous carrier or propellant useful for the formulation may include air, nitrogen and/or carbon dioxide.

[0039] A surfactant useful with the invention may include, but is not limited to, an alkyl sulfate, an alkylsulfonate, an alkylarylsulfonate, an alkyl aryl ether, a polyoxyethylene-alkyl aryl ether, a polyethylene glycol ether, a polyhydric alcohol ether, glycerine, polyethylene glycol, a protein, a lipo protein, a glycoprotein, a lipoglycoprotein, a plant oil, an animal oil and/or a sugar alcohol derivative.

[0040] Example sticking agents, the dispersing agent, and other auxiliaries or additives include casein, gelatin, polysaccharides such as starch, gum arabic, cellulose derivatives and alginic acid, lignin derivatives, bentonite, sugars and/or synthetic water-soluble polymers such as polyvinyl alcohol, polyvinylpyrrolidone and/or polyacrylic acid.

[0041] An example stabilizer can include PAP (acid isopropyl phosphate), BHT (2,6-di-tert-butyl-4-methyphenol), BHA (mixture of 2-tert-butyl-4-methoxyphenol and 3-tert-butyl-4-methoxyphenol), vegetable oils, mineral oils, surfactants, fatty acids and/or esters of fatty acids.

[0042] The compositions or formulations of the invention may be applied by any method known in the art including, for example, spraying, pouring, dipping, or applied using a cap fitted to the head, for example, for killing head lice eggs. The compositions or formulations may be in the form of concentrated liquids, solutions, suspensions, sprays, powders, and the like, formulated to deliver an ovicidal effective concentration of the composition.

[0043] The compositions and formulations of the invention may be used to kill any insect pest egg. In particular, the present invention is useful for killing lice eggs. Head lice present a multi-billion-dollar problem worldwide. Current control methods include the use of insecticides (typically, an insect nerve poison). These methods frequently fail because of resistance that has developed to the insecticide. Moreover, most products that have been developed kill larvae and adults but not the eggs (e.g., benzyl alcohol, pyrethrins and permethrins). Current treatments for killing all forms of lice are highly flammable and/or toxic and typically used only as a last resort to address highly resistant forms of lice (e.g., malthion, lidane). The present invention overcomes this problem by providing compositions and/or formulations that can kill all stages of lice development, adult, larval, and egg.

[0044] Accordingly, the present invention provides compositions comprising naturally occurring non-toxic products that surprisingly kill insect eggs. Thus, in some embodiments, the present invention provides a method of killing insect eggs, comprising contacting the insect pest eggs with an effective amount of a composition comprising 2-undecanone and/or 2-tridecanone, thereby killing the insect pest eggs. In other embodiments, a method of killing lice eggs (nits) is provided, comprising contacting the lice eggs with an effective amount of a composition comprising 2-undecanone and/or 2-tridecanone, thereby killing the lice eggs. Additionally provided is a method of killing lice eggs on a subject infested with lice eggs or suspected of being infested with lice eggs (e.g., a subject in need), comprising applying to the subject an effective amount of a composition comprising 2-undecanone and/or 2-tridecanone, thereby killing the lice eggs on the subject. The composition may comprise 2-undecanone or 2-tridecanone or a combination of 2-undecanone and 2-tridecanone in various insecticidal and/or ovidical ratios. In representative embodiments, the composition may further comprise DE.

[0045] As used herein, "a subject infested with lice eggs" or "suspected of being infested with lice eggs" may have one or more lice eggs on their person (head, scalp, hair, eye lashes, eye brows).

[0046] In some embodiments, the formulation comprising 2-undecanone and 2-tridecanone and DE can be used to prevent or reduce the occurrence or risk of infestation and/or re-infestation by an insect pest. Thus, in some embodiments, a method for preventing or reducing risk of infestation and/or re-infestation by an insect pest (e.g., lice, head lice, body lice) is provided comprising contacting the insect pest eggs with an effective amount of a composition comprising 2-undecanone and/or 2-tridecanone, and DE and optionally oil, thereby killing the insect pest eggs and preventing or reducing the risk of infestation and/or re-infestation by the insect pest. For example, the risk of infestation or re-infestation by lice can be reduced because the 2-undecanone and 2-tridecanone (with/without and DE) kill the eggs and while the 2-undecanone and 2-tridecanone may evaporate, the DE that is left behind kills any lice that may be picked up after treatment of a prior infestation. The effectiveness at reducing the risk of infestation or re-infestation can be further improved by the addition of oil(s) (or other binders/carriers) to the formulation (e.g., plant based oils), which reduce the volatility of the 2-undecanone and 2-tridecanone, thereby providing additional time for the compounds to kill the insect pest eggs as compared to a formulation that does not comprise the oils (or other binders/carriers). In some embodiments, increasing the ratio of 2-tridecanone to 2-undecanone (e.g., 3:1) can decrease the evaporation of the formulation, and therefore, increases the time that the therapeutic effect (egg killing) of the formulation is retained from hours to days as compared to a formulation that does not comprise a higher ratio of 2-tridecanone to 2-undecanone.

[0047] A formulation for reducing infestation or re-infestation can be therapeutically effective for at least about one hour to about seven days (e.g., about 1 hour, 2 hours, 3 hours, 4 hours, 5 hours, 6 hours, 7 hours, 8 hours, 9 hours, 10 hours, 12 hours, 15 hours, 20 hours, 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, and the like, or any range or value therein). Thus, in some embodiments, a formulation for reducing infestation or re-infestation may be therapeutically effective for at least about 1 hour to about 4 hours, about 1 hour to about 6 hours, about 1 hour to about 12 hours, about 1 hour to about 18 hours, about 6 hours to about 12 hours, about 6 hours to about 18 hours, about 6 hours to

about 24 hours, about 6 hours to about 2 days, about 6 hours to about 4 days, about 12 hours to about 1 day, about 12 hours to about 6 days, about 1 day to about 6 days, about 1 day to about 5 days, about 1 day to about 4 days, about 1 day to about 3 days, about 1 day to about 2 days, about 2 days to about 6 days, about 2 days to about 5 days, about 2 days to about 4 days, about 2 days to about 3 days, and the like, or any range or value therein.

[0048] In some embodiments, the contacting or applying comprises covering a site of infestation on a subject having or suspected of having lice eggs (e.g., the scalp and hair) with a cap, wherein the cap comprises an inner side and an outer side, and the inner side is in contact with the site of infestation on the subject and comprises the composition; and massaging the cap while the cap is on the site of infestation, thereby massaging the composition into the subject's hair and scalp. The composition may be provided in the cap in a liquid or a dry formulation that is massaged into the hair and scalp. Alternatively or in addition, in some embodiments, the composition may be formulated as a gas or aerosol, which is introduced into the cap when the cap covers the site of infestation.

[0049] The concentration of the 2-undecanone and/or 2-tridecanone will vary depending upon the nature of the particular formulation, as for example, whether it is a concentrate or to be used directly. Thus, for example, the 2-undecanone and/or 2-tridecanone will be present in the composition in a concentration of at least about 0.01% by weight to about 100% by weight of the total composition (e.g., about 0.01, 0.05, 0.1, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 96, 97, 98, 99, 99.9, 100% and any value or range therein). A carrier may be from 0.1% to 99.999% by weight of the total composition (e.g., about 0.1, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 96, 97, 98, 99, 99.9, 99.99, 99.999% or any value or range therein). In some embodiments, an ovicidal effective amount of the composition can comprise about 0.01% to about 50%, about 0.01% to about 40%, about 0.01% to about 30%, about 0.01% to about 20%, about 0.01% to about 15%, about 0.01% to about 10%, about 0.01% to about 8%, about 0.1% to about 50%, about 0.1% to about 40%, about 0.1% to about 30%, about 0.1% to about 20%, about 0.1% to about 15%, about 0.1% to about 10%, about 0.1% to about 8%, about 1% to about 50%, about 1% to about 40%, about 1% to about 30%, about 1% to about 20%, about 1% to about 15%, about 1% to about 10%, about 1% to about 8% (w/w in solid formulations and w/v in liquid formulations) of the total amount of 2-undecanone and/or 2-tridecanone and any range or value therein.

[0050] In some embodiments, when a composition or formulation of the invention comprises both 2-undecanone and 2-tridecanone, the 2-undecanone and/or 2-tridecanone may be present in a ratio from 0.01:20 to 20:0.01 of. In some embodiments, the ratio of 2-undecanone:2-tridecanone can be 6:1, 5:1, 4:1.3:1, 2:1, 1:1, 1:2, 1:3, 1:4, 1:5, 1:6, and the like and any range or value therein. In representative embodiments, the ratio of 2-undecanone:2-tridecanone can be 1:3. In some embodiments, a composition or formulation of the invention may comprise one or more additional methyl ketones, wherein R=C7, C8, C11-C20, or any combination thereof.

[0051] In some embodiments, the formulation of 2-undecanone and/or 2-tridecanone can comprise diatomaceous earth (DE) in an amount of about 0.1% to about 99.9%. Thus, in some embodiments the formulation comprises about 0.1% 2-undecanone and/or 2-tridecanone and about 1% DE, about 0.5% 2-undecanone and/or 2-tridecanone and about 1% DE, about 1% 2-undecanone and/or 2-tridecanone and about 1% DE, about 5% 2-undecanone and/or 2-tridecanone and about 1% DE, about 8% 2-undecanone and/or 2-tridecanone and about 1% DE, about 10% 2-undecanone and/or 2-tridecanone and about 1% DE, about 15% 2-undecanone and/or 2-tridecanone and about 1% DE, about 20% 2-undecanone and/or 2-tridecanone and about 1% DE, about 0.1% 2-undecanone and/or 2-tridecanone and about 5% DE, about 0.5% 2-undecanone and/or 2-tridecanone and about 5% DE, about 1% 2-undecanone and/or 2-tridecanone and about 5% DE, about 5% 2-undecanone and/or 2-tridecanone and about 5% DE, about 8% 2-undecanone and/or 2-tridecanone and about 5% DE, about 10% 2-undecanone and/or 2-tridecanone and about 5% DE, about 15% 2-undecanone and/or 2-tridecanone and about 5% DE, about 20% 2-undecanone and/or 2-tridecanone and about 5% DE, about 0.1% 2-undecanone and/or 2-tridecanone and about 10% DE, about 0.5% 2-undecanone and/or 2-tridecanone and about 10% DE, about 1% 2-undecanone and/or 2-tridecanone and about 10% DE, about 5% 2-undecanone and/or 2-tridecanone and about 10% DE, about 8% 2-undecanone and/or 2-tridecanone and about 10% DE, about 10% 2-undecanone and/or 2-tridecanone and about 10% DE, about 15% 2-undecanone and/or 2-tridecanone and about 10% DE, about 20% 2-undecanone and/or 2-tridecanone and about 10% DE, about 0.1% 2-undecanone and/or 2-tridecanone and about 15% DE, about 0.5% 2-undecanone and/or 2-tridecanone and about 15% DE, about 1% 2-undecanone and/or 2-tridecanone and about 15% DE, about 5% 2-undecanone and/or 2-tridecanone and about 15% DE, about 8% 2-undecanone and/or 2-tridecanone and about 15% DE, about 10% 2-undecanone and/or 2-tridecanone and about 15% DE, about 15% 2-undecanone and/or 2-tridecanone and about 15% DE, about 20% 2-undecanone and/or 2-tridecanone and about 15% DE, about 0.1% 2-undecanone and/or 2-tridecanone and about 20% DE, about 0.5% 2-undecanone and/or 2-tridecanone and about 20% DE, about 1% 2-undecanone and/or 2-tridecanone and about 20% DE, about 5% 2-undecanone and/or 2-tridecanone and about 20% DE, about 8% 2-undecanone and/or 2-tridecanone and about 20% DE, about 10% 2-undecanone and/or 2-tridecanone and about 20% DE, about 15% 2-undecanone and/or 2-tridecanone and about 20% DE, about 20% 2-undecanone and/or 2-tridecanone and about 20% DE, about 0.1% 2-undecanone and/or 2-tridecanone and about 25% DE, about 0.5% 2-undecanone and/or 2-tridecanone and about 25% DE, about 1% 2-undecanone and/or 2-tridecanone and about 25% DE, about 5% 2-undecanone and/or 2-tridecanone and about 25% DE, about 8% 2-undecanone and/or 2-tridecanone and about 25% DE, about 10% 2-undecanone and/or 2-tridecanone and about 25% DE, about 15% 2-undecanone and/or 2-tridecanone and about 25% DE, and/or about 20% 2-undecanone and/or 2-tridecanone and about 25% DE. In representative embodiments, the formulation of 2-undecanone and/or 2-tridecanone can comprise a ratio of 2-undecanone:2-tridecanone of 1:3 and diatomaceous earth (DE) in an amount of about 0.1% to about 99.9%. In some embodiments, a formulation can further comprise oils and/or other carriers to reduce the evaporation of the 2-undecanone and/or 2-tridecanone, thereby retaining the therapeutic effect (egg killing)

of the formulation over a period of time from hours to days (e.g., at least about one hour to about seven days (e.g., at least about 1 hour, 2 hours, 3 hours, 4 hours, 5 hours, 6 hours, 7 hours, 8 hours, 9 hours, 10 hours, 12 hours, 15 hours, 20 hours, 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, about 1 hour to about 4 hours, about 1 hour to about 6 hours, about 1 hour to about 12 hours, about 1 hour to about 18 hours, about 6 hours to about 12 hours, about 6 hours to about 18 hours, about 6 hours to about 24 hours, about 6 hours to about 2 days, about 6 hours to about 4 days, about 12 hours to about 1 day, about 12 hours to about 6 days, about 1 day to about 6 days, about 1 day to about 5 days, about 1 day to about 4 days, about 1 day to about 3 days, about 1 day to about 2 days, about 2 days to about 3 days, about 2 days to about 4 days, about 2 days to about 5 days, about 2 days to about 6 days, and the like, or any range or value therein).

[0052] In some embodiments, a formulation of 2-undecanone and/or 2-tridecanone may comprise a solvent, for example, ethanol. Thus, for example, a formulation of the invention may comprise about 0.01% to about 99.9% ethanol. Thus, in some embodiments the formulation comprises about 0.1% 2-undecanone and/or 2-tridecanone and about 1% to about 99.9% ethanol; about 0.5% 2-undecanone and/or 2-tridecanone and about 1% to about 99.5% ethanol, about 1% 2-undecanone and/or 2-tridecanone and about 1% to about 99% ethanol; about 5% 2-undecanone and/or 2-tridecanone and about 1% to about 95% ethanol; about 8% 2-undecanone and/or 2-tridecanone and about 1% to about 92% ethanol; about 10% 2-undecanone and/or 2-tridecanone and about 1% to about 90% ethanol; about 15% 2-undecanone and/or 2-tridecanone and about 1% to about 85% ethanol; about 20% 2-undecanone and/or 2-tridecanone and about 1% to about 80% ethanol; or about 25% 2-undecanone and/or 2-tridecanone and about 1% to about 75% ethanol; and the like.

[0053] In some embodiments, the formulation of 2-undecanone and/or 2-tridecanone can comprise diatomaceous earth (DE) and a solvent, such as ethanol. Thus, in some embodiments the formulation comprises about 0.1% 2-undecanone and/or 2-tridecanone, about 1% DE, and about 1% to about 98.9% ethanol; about 0.5% 2-undecanone and/or 2-tridecanone, about 1% DE, and about 1% to about 98.5% ethanol; about 1% 2-undecanone and/or 2-tridecanone, about 1% DE, and about 1% to about 98% ethanol; about 5% 2-undecanone and/or 2-tridecanone, about 1% DE, and about 1% to about 94% ethanol; about 8% 2-undecanone and/or 2-tridecanone, about 1% DE, and about 1% to about 91% ethanol; about 10% 2-undecanone and/or 2-tridecanone, about 1% DE, and about 1% to about 89% ethanol; about 15% 2-undecanone and/or 2-tridecanone, about 1% DE, and about 1% to about 84% ethanol; about 20% 2-undecanone and/or 2-tridecanone, about 1% DE, and about 1% to about 79% ethanol; about 0.1% 2-undecanone and/or 2-tridecanone, about 5% DE, and about 1% to about 94.9% ethanol; about 0.5% 2-undecanone and/or 2-tridecanone, about 5% DE, and about 1% to about 94.5% ethanol; about 1% 2-undecanone and/or 2-tridecanone, about 5% DE, and about 1% to about 94% ethanol; about 5% 2-undecanone and/or 2-tridecanone, about 5% DE, and about 1% to about 90% ethanol; about 8% 2-undecanone and/or 2-tridecanone, about 5% DE, and about 1% to about 87% ethanol; about 10% 2-undecanone and/or 2-tridecanone, about 5% DE, and about 1% to about 85% ethanol; about 15% 2-undecanone and/or 2-tridecanone, about 5% DE, and about 1% to about 80% ethanol; about 20% 2-undecanone and/or 2-tridecanone, about 5% DE, and about 1% to about 75% ethanol; about 0.1% 2-undecanone and/or 2-tridecanone, about 10% DE, and about 1% to about 90.9% ethanol; about 0.5% 2-undecanone and/or 2-tridecanone, about 10% DE, and about 1% to about 89.5% ethanol; about 1% 2-undecanone and/or 2-tridecanone, about 10% DE, and about 1% to about 89% ethanol; about 5% 2-undecanone and/or 2-tridecanone, about 10% DE, and about 1% to about 85% ethanol; about 8% 2-undecanone and/or 2-tridecanone, about 10% DE, and about 1% to about 82% ethanol; about 10% 2-undecanone and/or 2-tridecanone, about 10% DE, and about 1% to about 80% ethanol; about 15% 2-undecanone and/or 2-tridecanone, about 10% DE, and about 1% to about 75% ethanol; about 20% 2-undecanone and/or 2-tridecanone, about 10% DE, and about 1% to about 70% ethanol; about 0.1% 2-undecanone and/or 2-tridecanone, about 15% DE, and about 1% to about 84.9% ethanol; about 0.5% 2-undecanone and/or 2-tridecanone, about 15% DE, and about 1% to about 84.5% ethanol; about 1% 2-undecanone and/or 2-tridecanone, about 15% DE, and about 1% to about 84% ethanol; about 5% 2-undecanone and/or 2-tridecanone, about 15% DE, and and about 1% to about 80% ethanol; about 8% 2-undecanone and/or 2-tridecanone, about 15% DE, and about 1% to about 77% ethanol; about 10% 2-undecanone and/or 2-tridecanone, about 15% DE, and about 1% to about 75% ethanol; about 15% 2-undecanone and/or 2-tridecanone, about 15% DE, and about 1% to about 70% ethanol; about 20% 2-undecanone and/or 2-tridecanone, about 15% DE, and about 1% to about 65% ethanol; about 0.1% 2-undecanone and/or 2-tridecanone, about 20% DE, and about 1% to about 79.9% ethanol; about 0.5% 2-undecanone and/or 2-tridecanone, about 20% DE, and about 1% to about 79.5% ethanol; about 1% 2-undecanone and/or 2-tridecanone, about 20% DE, and about 1% to about 79% ethanol; about 5% 2-undecanone and/or 2-tridecanone, about 20% DE, and about 1% to about 75% ethanol; about 8% 2-undecanone and/or 2-tridecanone, about 20% DE, and about 1% to about 72% ethanol; about 10% 2-undecanone and/or 2-tridecanone, about 20% DE, and about 1% to about 70% ethanol; about 15% 2-undecanone and/or 2-tridecanone, about 20% DE, and about 1% to about 65% ethanol; about 20% 2-undecanone and/or 2-tridecanone, about 20% DE, and about 1% to about 60% ethanol; about 0.1% 2-undecanone and/or 2-tridecanone, about 25%DE, and about 1% to about 74.9% ethanol; about 0.5% 2-undecanone and/or 2-tridecanone, about 25% DE, and about 1% to about 74.5% ethanol; about 1% 2-undecanone and/or 2-tridecanone, about 25% DE, and about 1% to about 74% ethanol; about 5% 2-undecanone and/or 2-tridecanone, about 25% DE, and about 1% to about 70% ethanol; about 8% 2-undecanone and/or 2-tridecanone, about 25% DE, and about 1% to about 67% ethanol; about 10% 2-undecanone and/or 2-tridecanone, about 25% DE, and about 1% to about 65% ethanol; about 15% 2-undecanone and/or 2-tridecanone, about 25% DE, and about 1% to about 60% ethanol; about 20% 2-undecanone and/or 2-tridecanone, about 25% DE, and about 1% to about 55% ethanol, and the like. As described herein, a formulation of the invention may comprise other components including but not limited to, one or more essential oils (e.g., geraniol, lavender, lemon grass) and/or

other oils (e.g., cotton seed oil, vegetable oil, soybean oil, caster bean oil), and/or surfactants.

[0054] The applying or contacting of the compositions or formulation of the invention may be for a range of time (exposure time) from about 1 second to about 3 seconds, about 1 second to about 10 seconds, about 1 second to about 15 seconds, about 1 second to about 1 minute, about 1 second to about 5 minutes, about 1 second to about 15 minutes, about 1 second to about 1 hour, about 1 second to about 2 hours, about 1 second to about 6 hours, about 1 second to about 12 hours, about 1 second to about 18 hours, about 1 second to about 1 day, about 1 second to about 2 days, about 1 second to about 3 days, about 1 second to about 4 days, about 1 second to about 5 days, about 1 second to about 6 days, about 1 second to about 7 days, or more, and any range or value therein. Thus, in some embodiments, the time for applying may be about 1 second, 2 seconds, 3 seconds, 4 seconds, 5 seconds, 8 seconds, 9 seconds, 10 seconds, 15 seconds, 20 seconds, 30 seconds, 1 min, 2 min, 3 min, 4 min, 5 min, 6 min, 7 min, 8 min, 9 min, 10 min, 11 min, 12 min, 13 min, 14 min, 15 min, 20 min, 25 min, 30 mi, 1 hour, 2 hours, 3 hours, 4 hours, 5 hours, 6 hours, 7 hours, 8 hours, 9 hours, 10 hours, 12 hours, 15 hours, 20 hours, 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, and/or 7 days. In representative embodiments, the time for applying may be about 1 second to about 10 seconds. In particular embodiments, the time for applying may be about 15 minutes.

[0055] As well understood, the percentage of eggs killed may depend on the concentration of the ovicide and the exposure time to the ovicide (e.g., the time for applying). Thus, applying or contacting of the compositions and formulations of the invention generally results in the killing of about 45% to about 100% (e.g., about 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100%) of the insect pest eggs for any given time period. However, the compositions and formulations are shown to work very quickly and efficiently, resulting in a killing of 100% of the insect pest eggs following a very short contact or application time. Thus, in some embodiments, a one to five second exposure results in a 100% kill of the insect pest eggs. In some embodiments, exposure of insect pest eggs to a formulation of 1% 2-undecanone and 1% DE for 3 seconds kills about 100% of the insect pest eggs. In some embodiments, exposure of insect pest eggs to a formulation of 8% 2-undecanone and 1% DE for 1 second kills about 100% of the insect pest eggs. In some embodiments, exposure of insect pest eggs to a formulation of 5% 2-undecanone for 1 second kills about 70% of the insect pest eggs. In some embodiments, exposure of insect pest eggs to a formulation of 15% 2-undecanone for about one to about five second kills about 100% of the insect pest eggs. As discussed herein, the formulations of the invention once applied may be effective for a wide range of time from about six hours to about five days.

[0056] In addition to being useful for treating of animals (hair, skin, etc.), the compositions of the invention may also be applied to cloth, wood and other surfaces for control of insect pest eggs, including but not limited to, fabrics (clothing, linens, draperies, etc.), furniture (e.g., chairs, mattresses, box springs, etc.), carpets, containers, walls, base boards, floors, air, water, nets, paper (e.g., food packaging), trash containers, dumps, soil and/or plants.

[0057] In some embodiments, when used as an ovicide on a plant, the formulation may comprise an oil emulsion (e.g., a plant based oil such as cottonseed oil)

[0058] In some embodiments, the applying or contacting may be repeated as needed. Thus, in some embodiments, the applying or contacting may be repeated every six hours, every 12 hours, every 24 hours, every other day, every three days, once a week, and the like, as needed. Formulations comprising 2-undecanone and/or 2-tridecanone, and DE provide additional benefits with the DE providing longer term control of the insects, thereby reducing re-infestation.

[0059] The methods, compositions, and formulations described herein may be used to kill eggs from any insect pest including, but are not limited to, tick eggs, mite eggs, spider eggs, millipede eggs, centipede eggs, and nematode eggs. Example insect pests whose eggs may be killed using the compositions, and formulations described herein include the following:

[0060] Hemiptera: Delphacidae (planthoppers) such as *Laodelphax striatellus* (small brown planthopper), *Nilaparvata lugens* (brown planthopper) and *Sogatella furcifera* (white-backed rice planthopper); Cicadelloidea (leafhoppers) such as *Nephotettix cincticeps* (green rice leafhopper), *Nephotettix virescens* (green rice leafhopper) and *Recilia dorsalis;* Aphidoidea (aphids); stink bugs such as Pentatomidae, Acanthosomatidae, Urostylidae, Dinidoridae, Coreidae and Alydidae; Aleyrodidae(whiteflies); Tingidae (lace bugs); Psyllidae (jumping plantlice);

[0061] Lepidoptera: Pyralidae such as *Chilo suppressalis* (rice stem borer), *Cnaphalocrocis medinalis* (rice leafroller) and *Plodia interpunctella* (Indian meal moth); Noctuidae such as *Spodoptera litura* (tobacco cutworm), *Pseudaletia separata* (rice armyworm), *Mamestra brassicae* (cabbage armyworm); Pieridae such as *Pieris rapae crucivora* (common cabbageworm); Tortricidae such as *Adoxophyes* spp.; Carposinidae; Lyonetiidae; Lymantriidae (tussock moths); Plusiinae; *Agrotis* spp. such as *Agrotis segetum* and *Agrotis ipsilon* (black cutworm); *Heliotis* spp.; *Plutella xylostella* (diamondback moth); *Tinea pellionella* (casemaking clothes moth); *Tineola bisselliella* (webbing clothes moth);

[0062] Diptera: *Culex* spp. such as *Culex pipiens pallens* (common mosquito) and *Culex tritaeniorhynchus; Aedes* spp. such as *Aedes aegypti* and Aedes albopictus; *Anopheles* spp. such as *Anopheles sinensis;* Chironomidae (midges); Muscidae such as *Musca domestica* (housefly), *Muscina stabulans* (false stablefly) and *Fannia canicularis* (little housefly); Calliphoridae; Sarcophagidae; Anthomyiidae such as *Delia platura* (seedcorn maggot) and *Delia antiqua* (onion maggot); Tephritidae (fluit flies); Drosophilidae; Psychodidae (moth flies); Simuliidae (black flies); Tabanidae; Stomoxyidae; Ceratopogonidae (biting midges);

[0063] Coleoptera (beetles): *Diabrotica* spp. (corn rootworms) such as *Diabrotica virgifera* (western corn rootworm) and *Diabrotica undecimpunctata howardi* (southern corn rootworm); Scarabaeidae such as *Anomala cuprea* and *Anomala rufocuprea* (soybean beetle); Curculionidae such as *Sitophilus zeamais* (maize weevil) and *Lissorhoptrus oryzophilus* (ricewater weevil); Tenebrionidae (darkling beetles) such as *Tenebrio molitor* (yellow mealworm) and *Tribolium castaneum* (red flour beetle); Chrysomelidae such as Phyllotreta striolata (striped flea beetle) and *Aulacophora femoralis* (cucurbit leaf beetle); Anobiidae; *Epilachna* spp. such as *Epilachna vigintioctopunctata* (twentyeight-spotted ladybird); Lyctidae (powder post beetles); Bostrychidae (false powder post beetles); Cerambycidae; *Paederus fuscipes* (robe beetle);

[0064] Dictyoptera: *Blattella germanica* (German cockroach); *Periplaneta fuliginosa* (smokybrown cockroach); *Periplaneta americana* (American cockroach); *Periplaneta brunnea* (brown cockroach); *Blatta orientalis* (oriental cockroach);

[0065] Thysanoptera: *Thrips palmi; Thrips hawaiiensis* (flower thrips); thunderflies, thunderbugs, corn lice and other thrips;

[0066] Hymenoptera: Formicidae (ants); Vespidae (hornets); Bethylidae; Tenthredinidae (sawflies) such as *Athalis rosae ruficornis* (cabbage sawfly);

[0067] Orthoptera: Gryllotalpidae (mole crickets); Acrididae (grasshoppers);

[0068] Siphonaptera: *Ctenocephalides canis* (dog flea); *Ctenocephalides felis* (cat flea); *Pulex irritans;*

[0069] Anoplura: *Pediculus humanus capitis; Pediculus humanus corpori, Pthirus pubis;*

[0070] Isoptera: *Reticulitermes speratus; Coptotermes formosanus;*

[0071] Tetranychidae: *Tetranychus cinnabarinus* (carmine spider mite); *Tetranychus urticae* (two-spotted spider mite); *Tetranychus kanzawai* (Kanzawa spider mite); *Panonychus citri* (citrus red mite); *Panonychus ulmi* (European red mite);

[0072] House-dust mites: Acaridae; Dermatophagoidinae; Pyroglyphinae; Cheyletidae; Macronyssidae such as *Ornithonyssus* spp.;

[0073] Ticks: Ixodidae such as *Boophilus microplus;* and

[0074] Termites, including drywood, subterranean termites (or ground termites, including Eastern, Western, and Desert termites), and Formosan termites (sometimes referred to as Formosan subterranean termites or the "super termite").

[0075] The term "lice" or "louse" as used herein includes any type of "lice" or "louse," including, but not limited to, head lice, (*Pediculus humanus capitis*), body lice (*Pediculus humanus corporis*), and/or crab (pubic) lice (*Pthirus pubis*).

[0076] The invention will now be described with reference to the following examples. It should be appreciated that these examples are not intended to limit the scope of the claims to the invention, but are rather intended to be exemplary of certain embodiments. Any variations in the exemplified methods that occur to the skilled artisan are intended to fall within the scope of the invention.

EXAMPLES

Example 1

Killing Body Lice Eggs

[0077] Small, roughly equal-sized body lice nit swatches (25 to 100 eggs/replicate) were dipped into treatment solutions for five seconds using forceps. The treatments included 100% ethanol and a 15% solution of 2-undecanone (NC0728). The experiments included a no treatment control. The treated and control swatches were maintained at 27° C. and relative humidity of 76% in an insectary for duration of experiment. The number of lice that emerged were counted after 24 hours and 45 hours, with the count being cumulative over 45 hours total. The results are shown in FIGS. **1**A-**1**B. One louse died in the ethanol treatment.

Example 2

Killing Insect Eggs Using a Cotton Bollworm Model System

[0078] Newly oviposited eggs of the cotton bollworm were used as model insect eggs to examine the synergism between 2-undecanone and DE from a commercial source (Sigma, DE with no insecticidal activity). Due to their obligate parasitic lifestyle, lice, head lice, in particular, are very difficult to obtain. Thus, bollworm eggs were used in place of lice eggs for the experiments showing efficacy of the treatments described herein. Notably, bollworm eggs are understood to be more robust than lice eggs as a result of their method of deposition on leaves, which requires that they survive direct sunlight, wind, rain, predation and parasites. Therefore, it is understood that if the treatment works on bollworm eggs, it will also work on the eggs of head lice. Further, the experiments using body lice, a recognized proxy for head lice, confirmed the effectiveness of the treatment with the formulations of this invention.

[0079] All treatments were conducted by dipping the bollworm eggs in the different treatments for 3 seconds, allowing the eggs to dry at room temperature, and then assessing egg hatch for the following 4 days. The eggs typically hatch under these conditions in 2.5 days. The solvent used in these experiments was absolute ethanol.

[0080] FIG. **2** shows that a dose response was obtained with increasing amounts of 2-undecanone (from 1 to 8%), resulting in an increase in egg mortality to very high levels (up to about 90% under the testing conditions). The combination of 2-undecanone with DE at 1% enhanced the activity of the natural product. Alone, the DE used here was shown not to be insecticidal.

[0081] Thus, these data show that not only are the compositions of the invention effective for killing bollworm eggs but show that the bollworm egg system can be used effectively as a model system for lice eggs.

Example 3

Synergistic Effect of 2-undecanone and DE on Bollworm Egg Hatching

[0082] Sheets were cut with approximately 25 bollworm eggs per sheet. Three replicates of two solutions were tested: (1) 1% 2-undecanone in ethanol or (2) 1% 2-undecanone/1% DE (Thomas Labs DE) in ethanol.

[0083] Eggs were dipped in solutions for 1 second and allowed to dry for 10 minutes at room temperature before being placed in sealed petri dishes. Eggs were stored at 76 ° F. and hatching monitored every 24 hours for 4 days. The results are shown in FIG. **3**.

[0084] FIG. **3** shows the synergism between 1% 2-undecanone and DE as the 2-undecanone with DE was much more effective at reducing egg hatching when compared to 1% undecanone alone.

Example 4

Effect of Diatomaceous Earth (DE) on Bollworm Egg Hatching

[0085] Sheets were cut with approximately 25 bollworm eggs per sheet. Four replicates of three solutions were tested: (1) Untreated; (2) 1% Thomas Labs DE or (3) 1% Sigma DE.

[0086] Eggs were dipped in solutions for 1 second and allowed to dry for 10 minutes at room temperature before being placed in sealed petri dishes. Eggs were stored at 76 ° F. and hatching monitored every 24 hours for 4 days. The results are provided in FIG. **4** and show that DE alone has the same effect on bollworm egg hatching as no treatment at all.

[0087] 2-undecanone has been approved by the US EPA for use on human skin at 7.75% without restrictions (including children) and is shown to kill all stages of the lice. While the mechanical insecticide (DE) has no inherent ovicidal activity, it appears to synergize the ovicidal activity of 2-undecanone. Notably, the 2-undecanone results in minimal residual activity against eggs (typically, it evaporates in 30 min or less). The remaining mechanical insecticide (DE) persists to continue controlling the larval and adult stages for as long as the application site (e.g., head) is not washed. Thus, this residual activity of the mechanical insecticide can reduce re-infestation by killing any insect pests that may later attempt to re-infest the site.

[0088] The foregoing is illustrative of the present invention, and is not to be construed as limiting thereof. The invention is defined by the following claims, with equivalents of the claims to be included therein.

1. A method of killing insect eggs, comprising contacting the insect eggs with an effective amount of a composition comprising 2-undecanone and/or 2-tridecanone, thereby killing the insect eggs.

2. The method of claim **1**, wherein the insect eggs are lice eggs.

3. A method of killing lice eggs on a subject infested with lice eggs or suspected of being infested with lice eggs, comprising applying to the subject an effective amount of a composition comprising 2-undecanone and/or 2-tridecanone, thereby killing the lice eggs on the subject.

4. A method of reducing the risk of infestation or re-infestation of a subject by lice, comprising applying to the subject an effective amount of a composition comprising 2-undecanone and/or 2-tridecanone, thereby killing the lice eggs on the subject.

5. The method of claim **1**, wherein the composition comprises about 0.01% to about 30% of 2-undecanone and/or about 0.01% to about 30% of 2-tridecanone.

6. The method of claim **1**, wherein the composition further comprises a solid carrier, a liquid carrier, or a gaseous carrier.

7. The method of claim **6**, wherein the solid carrier is a powder or granules of cellulose, clays, talc, ceramics, and/or other inorganic minerals.

8. The method of claim **1**, wherein the composition further comprises diatomaceous earth.

9. The method of claim **8**, wherein the composition comprises about 1% to about 99.99% diatomaceous earth.

10. The method of claim **6**, wherein the liquid carrier is water, a surfactant, an oil, an organic solvent, and/or any combination thereof.

11. The method of claim **10**, wherein the surfactant is an alkyl sulfate, alkylsulfonate, alkylarylsulfonate, alkyl aryl ether, polyoxyethylenealkyl aryl ether, polyethylene glycol ether, polyhydric alcohol ether, sugar alcohol derivative, or any combination thereof.

12. The method of claim **10**, wherein the organic solvent is ethanol and/or methanol.

13. The method of claim **6**, wherein the gaseous carrier is air, carbon dioxide, and/or nitrogen.

14. The method of claim **1**, wherein the composition is in the form of a powder, liquid, aerosol, gel, foam, lotion, or ointment.

15. The method of claim **1**, wherein the killing occurs in at least about 1 second to about 10 seconds following the contacting of the insect eggs and/or lice eggs with the effective amount of the composition or after applying to the subject the effective amount of the composition, resulting in the killing of about 45% to about 100% of the insect eggs and/or lice eggs.

16. The method of claim **1**, wherein the contacting comprises contacting a site of lice infestation on a subject with a dry shampoo, liquid shampoo, conditioner, creme rinse, mousse, foam, lotion, ointment, and/or aerosol comprising the composition.

17. The method of claim **3**, wherein the composition is applied to the subject in the form of a dry shampoo, liquid shampoo, conditioner, creme rinse, mousse, foam, lotion, ointment, and/or aerosol that comprises the composition.

18. The method of claim **1**, wherein the contacting comprises:

covering a site of infestation on a subject having or being suspected of having lice eggs with a cap, wherein the cap comprises an inner side and an outer side, and the inner side is in contact with the site of infestation on the subject and comprises the composition; and

massaging the cap while the cap is on the site of infestation, thereby massaging the composition into the subject's hair and scalp.

19. The method of claim **3**, wherein the applying comprises:

covering a site of infestation on a subject having or being suspected of having lice eggs with a cap, wherein the cap comprises an inner side and an outer side, and the inner side is in contact with the site of infestation on the subject and comprises the composition; and

massaging the cap while the cap is on the site of infestation, thereby massaging the composition into the subject's hair and scalp.

20. The method of claim **3**, wherein the composition that is applied to the subject or contacted with the eggs is therapeutically effective for at least about six hours to about five days.

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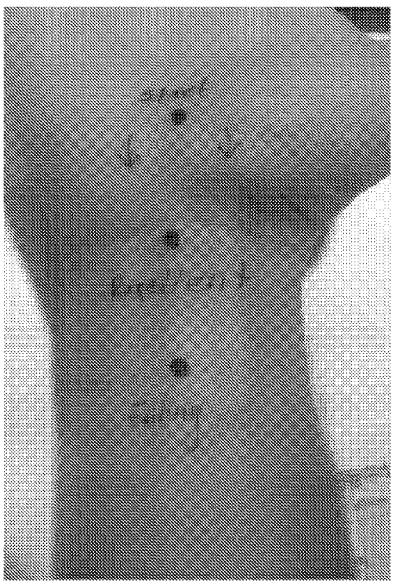
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(54) **Title:** INSECT AND TICK REPELLENT FORMULATIONS AND METHODS OF USE THEREOF

(57) Abstract: The invention relates to a formulation comprising: 2-undecanone in an amount of about 5% to about 50%; at least one hydrophobic base in an amount of about 5% to about 65%; at least one alcohol in an amount of about 5% to about 50%; and at least one emulsifier in an amount of about 5% to about 15% and methods of repelling insects and ticks by applying to a subject or a substrate a formulation of the invention.

Fig. 1



WO 2019/133675 A1

INSECT AND TICK REPELLENT FORMULATIONS AND METHODS OF USE THEREOF

STATEMENT OF PRIORITY

This application claims the benefit, under 35 U.S.C. § 119 (e), of U.S. Provisional Application No. 62/61 1,178 filed on December 28, 2017, the entire contents of which is incorporated by reference herein.

FIELD OF THE INVENTION

The present invention concerns methods and formulations for repelling insects and ticks.

BACKGROUND OF THE INVENTION

Insect and tick repellants are widely used throughout the United States and throughout the world. In some regions, the use of repellants is important for avoiding or reducing the occurrence of diseases carried by insects and ticks. For example, the Centers for Disease Control (CDC) receives nearly 10,000 reports of Lyme disease (transmitted by deer ticks) and 1,000 reports of encephalitis (transmitted by mosquitoes) annually.

Currently, the most common repellent is N,N-diethyl-meta-toluamide (DEET). DEET was designed to be applicable to the skin of subjects, and is designed to repel rather than kill insects and ticks. Although in use for some time, concern has recently emerged about the potential toxicity of DEET to children. Recently the US Environmental Protection Agency (EPA) determined that it would no longer allow child safety claims on labels for DEET-containing products.

Improved insect and tick repellants useful for all persons are needed.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 provides photograph of a representative arm with descriptive labels to show the set-up for testing tick foraging. The labels were not present during actual testing.

SUMMARY OF THE INVENTION

A first aspect of the invention provides a formulation comprising: 2-undecanone in an amount of about 5% to about 50% by volume; at least one hydrophobic base in an amount of about 5% to about ***65%*** by volume; at least one alcohol in an amount of about 5% to about

50% by volume; and at least one emulsifier in an amount of about 5% to about 15% by volume.

A second aspect provides a formulation comprising: 2-undecanone in an amount of about 5% to about 30% by volume; at least one hydrophobic base in an amount of about 25% to about 40% by volume; at least one alcohol in an amount of about 25% to about 40% by volume; and at least one emulsifier in an amount of about 5% to about 15% by volume.

A third aspect of the invention provides a formulation comprising: 2-undecanone in an amount of about 10% to about 25% by volume; at least one hydrophobic base in an amount of about 25% to about 35% by volume; at least one alcohol in an amount of about 25% to about 40% by volume; and at least one emulsifier in an amount of about 5% to about 15% by volume.

A fourth aspect of the invention provides a method of repelling an insect pest or a tick, comprising: applying to a subject a formulation of the present invention in an amount effective to repel the insect or tick.

DETAILED DESCRIPTION

The present invention now will be described hereinafter with reference to the accompanying drawings and examples, in which embodiments of the invention are shown. This description is not intended to be a detailed catalog of all the different ways in which the invention may be implemented, or all the features that may be added to the instant invention. For example, features illustrated with respect to one embodiment may be incorporated into other embodiments, and features illustrated with respect to a particular embodiment may be deleted from that embodiment. Thus, the invention contemplates that in some embodiments of the invention, any feature or combination of features set forth herein can be excluded or omitted. In addition, numerous variations and additions to the various embodiments suggested herein will be apparent to those skilled in the art in light of the instant disclosure, which do not depart from the instant invention. Hence, the following descriptions are intended to illustrate some particular embodiments of the invention, and not to exhaustively specify all permutations, combinations and variations thereof.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. The terminology used in the description of the invention herein is for the purpose of describing particular embodiments only and is not intended to be limiting of the invention.

All publications, patent applications, patents and other references cited herein are incorporated by reference in their entireties for the teachings relevant to the sentence and/or paragraph in which the reference is presented.

Unless the context indicates otherwise, it is specifically intended that the various features of the invention described herein can be used in any combination. Moreover, the present invention also contemplates that in some embodiments of the invention, any feature or combination of features set forth herein can be excluded or omitted. To illustrate, if the specification states that a composition comprises components A, B and C, it is specifically intended that any of A, B or C, or a combination thereof, can be omitted and disclaimed singularly or in any combination.

As used in the description of the invention and the appended claims, the singular forms "a," "an" and "the" are intended to include the plural forms as well, unless the context clearly indicates otherwise.

Also, as used herein, "and/or" refers to and encompasses any and all possible combinations of one or more of the associated listed items, as well as the lack of combinations when interpreted in the alternative ("or").

The term "about," as used herein when referring to a measurable value such as a dosage or time period and the like refers to variations of ± 10%, ± 5%, ± 1%, ± 0.5%, or even ± 0.1% of the specified amount.

As used herein, phrases such as "between X and Y" and "between about X and Y" should be interpreted to include X and Y. As used herein, phrases such as "between about X and Y" mean "between about X and about Y" and phrases such as "from about X to Y" mean "from about X to about Y."

The term "comprise," "comprises" and "comprising" as used herein, specify the presence of the stated features, integers, steps, operations, elements, and/or components, but do not preclude the presence or addition of one or more other features, integers, steps, operations, elements, components, and/or groups thereof.

As used herein, the transitional phrase "consisting essentially of' means that the scope of a claim is to be interpreted to encompass the specified materials or steps recited in the claim and those that do not materially affect the basic and novel characteristic(s) of the claimed invention. Thus, the term "consisting essentially of' when used in a claim of this invention is not intended to be interpreted to be equivalent to "comprising."

As used herein, the terms "increase," "increasing," "increased," "enhance," "enhanced," "enhancing," and "enhancement" (and grammatical variations thereof) describe an elevation of at least about 25%, 50%, 75%, 100%, 150%, 200%, 300%, 400%, 500% or more as compared to a control.

As used herein, the terms "reduce," "reduced," "reducing," "reduction," "diminish," and "decrease" (and grammatical variations thereof), describe, for example, a decrease of at least about 5%, 10%, 15%, 20%, 25%, 35%, 50%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 100% as compared to a control. In particular embodiments, the reduction can result in no or essentially no *(i.e.,* an insignificant amount, *e.g.,* less than about 10% or even 5%) detectable activity or amount.

The present invention provides monophasic formulations comprising undecanone for use in repelling insects and/or ticks. Thus, in some embodiments, the formulations of the invention are not in the form of emulsions but are in a single phase.

In some embodiments, the formulations may be in the form of an emulsion that is homogeneous.

In some embodiments, the formulations of the invention may be used to repel animals generally including, but not limited to, insects, ticks, spiders, hunting spiders, and scorpions.

Notably, 2-undecanone alone is not an effective insect or tick repellent for at least the reasons that it evaporates too fast and its duration of repellency is less than about 30 min. The formulations of the present invention comprising 2-undecanone, at least one hydrophobic base (for example, a seed oil), at least one alcohol, and at least one emulsifier provide significant repellency lasting over many hours after application to a subject.

Accordingly, in some embodiments, the present invention provides a formulation comprising: 2-undecanone in an amount of about 5% to about 50% by volume (e.g., about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 50%, or any range or value therein); at least one hydrophobic base in an amount of about 5% to about 65% by volume (e.g., about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 50%, 55%, 60%, 65%, or any range or value therein); at least one alcohol in an amount of about 5% to about 50% by volume (e.g., about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 50%, or any range or value therein); and at least one emulsifier(i.e., total amount of all emulsifiers) in an amount of about 5% to about 15% by volume (e.g., about 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, or any range or value therein). In some embodiments, the total amount of hydrophobic base in the formulation may be in a range from about 5%-65% by volume of the formulation. In some embodiments, the total amount of alcohol in the formulation may be in a range from about

5% to about 50% by volume of the formulation. In some embodiments, the total amount of emulsifier in the formulation may be in a range from about 5% to about 15% by volume of the formulation.

In some embodiments, a formulation is provided comprising: 2-undecanone in an amount of about 5% to about 30% by volume (e.g., about 5%, 10%, 15%, 20%, 25%, 30%, or any range or value therein); at least one hydrophobic base in an amount of about 25% to about 40% by volume (e.g., about 25%, 26%, 27%, 28%, 29%, 30%, 13%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, or any range or value therein); at least one alcohol in an amount of about 25% to about 40% by volume (e.g., about 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40% by volume, or any range or value therein); and at least one emulsifier (i.e., total amount of all emulsifiers) in an amount of about 5% to about 15% by volume or about 5% to about 15% by volume (e.g., about 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, or any range or value therein). In some embodiments, the total amount of hydrophobic base in the formulation may be in a range from about 25%-40% by volume of the formulation. In some embodiments, the total amount of alcohol in the formulation may be in a range from about 25% to about 40% by volume of the formulation. In some embodiments, the total amount of emulsifier in the formulation may be in a range from about 5% to about 15% by volume of the formulation.

In some embodiments, the present invention provides a formulation comprising: 2-undecanone in an amount of about 10% to about 25% by volume (e.g., about 10%, 15%, 20%, 21%, 22%, 23%, 24%, 25%, or any range or value therein; e.g., about 12% to about 16%, about 12% to about 17%, about 13% to about 16%, about 13% to about 17%, about 14% to about 16% by volume, or any range or value therein); at least one hydrophobic base in an amount of about 25% to about 35% by volume (e.g., about 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35%, or any range or value therein; e.g., about 26% to about 33%, about 26% to about 35%, about 28% to about 32%, about 28% to about 33%, about 28% to about 35%,%, or any range or value therein); at least one alcohol in an amount of about 25% to about 40% by volume (e.g., about 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, or any range or value therein; e.g., about 30% to about 38%, about 30% to about 40%, about 32% to about 37%, about 32% to about 38%, about 33% to about 37%, about 33% to about 36%, or any range or value therein); and at least one emulsifier (i.e., total amount of all emulsifiers) in an amount of about 5% to about 15% by volume or about 5% to about 15% by volume (e.g., about 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, or any range or value therein; e.g., about 7% to about

12%, about 7% to about 13%, about 7% to about 14%, about 8% to about 11%, about 8% to about 12%, about 8% to about 13%, or any range or value therein). In some embodiments, a formulation is provided comprising 2-undecanone in an amount of about 10% to about 20% by volume (e.g., about 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, or any range or value therein); at least one hydrophobic base in an amount of about 30% to about 40% by volume (e.g., about 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, or any range or value therein); at least one alcohol in an amount of about 30% to about 40% by volume (e.g., about 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, or any range or value therein); and at least one emulsifier (i.e., total amount of all emulsifiers) in an amount of about 5% to about 15% by volume (e.g., about 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, or any range or value therein). In some embodiments, the total amount of hydrophobic base in the formulation may be in a range from about 25%-35% by volume of the formulation or about 30% to about 40% by volume of the formulation. In some embodiments, the total amount of alcohol in the formulation may be in a range from about 25% to about 40% by volume of the formulation or about 30% to about 40% by volume of the formulation. In some embodiments, the total amount of emulsifier in the formulation may be in a range from about 5% to about 15% by volume of the formulation.

2-undecanone (also known as methyl nonyl ketone) comprises the structure of



(I)

wherein R is C_9.

The formulations of the invention comprise a hydrophobic base. “Hydrophobic base" as used herein refers to a natural and/or synthetic fat, oil, and/or the like. Any suitable hydrophobic base may be used in a formulation of the present invention. In certain embodiments of the present invention, a formulation may comprise two or more hydrophobic bases, such as, but not limited to, 2, 3, 4, 5, or more hydrophobic bases. Exemplary hydrophobic bases include, but are not limited to, branched and unbranched hydrocarbons, hydrophobic polymers, fatty acids, fatty acid esters, monoglycerides, diglycerides, and/or triglycerides. A hydrophobic base may be partially or wholly saturated and/or unsaturated.

Additional exemplary hydrophobic bases may include, but are not limited to plant and/or vegetable oils, animal oils, and/or mineral oil. A plant-based oil may be any oil obtained from a plant or plant part (e.g., oil from seeds, flowers, fruit, leaves, bark, roots, etc). Exemplary plant oils include avocado oil, borage oil, canola (rapeseed) oil, castor oil,

chamomile oil, coconut oil, corn oil, cottonseed oil, rapeseed oil, evening primrose oil, safflower oil, sunflower oil, soybean oil, sweet almond, palm oil, palm kernel oil, arctium lappa seed oil, sesame oil, borgo officialis seed oil, brassica campestris oleifera oil, brevoortia oil, bubulum oil, cistus ladaniferus oil, elaeis guineensis oil, almond oil, pine oil, olive oil, peanut oil, flax seed oil, wheat germ oil, hemp oil, jojoba oil, linseed oil, macadamia oil, neem oil, argan oil, grape seed oil, thistle oil, and/or algal oil and/or any combination thereof.

In some embodiments, the molecular weight of a hydrophobic base useful with this invention may range from about 100 g/mol to about 1000 g/mol. Thus, in some embodiments, the molecular weight of a hydrophobic base useful with this invention may range from about 100 g/mol to about 750 g/mol, about 150 g/mol to about 750 g/mol, about 150 g/mol to about 500, about 150 g/mol to about 300g/mol, and any range or value therein.

In some embodiments, a hydrophobic base may be a 16 carbon or 18 carbon compound or a combination thereof. In some embodiments, a hydrophobic base may be about 10-20% saturated. In some embodiments, a hydrophobic base may be about 75-90% unsaturated. In some embodiments, a hydrophobic base may have a density of about 0.91 to 0.93 g/cm3 between the temperatures of 15 °C and 25 °C

In some embodiments, a formulation of the invention comprises at least one hydrophobic base that is a plant-based oil. In some embodiments, the at least one plant-based oil of a formulation of the invention may comprise, consist essentially of, or consist of soybean oil. In some embodiments, the at least one seed oil of a formulation of the invention may comprise, consist essentially of, or consist of cottonseed oil. In some embodiments, the at least one seed oil of a formulation of the invention may comprise, consist essentially of coconut oil. In some embodiments, the at least one seed oil of a formulation of the invention may comprise, consist essentially of, or consist of soybean oil and cottonseed oil. In some embodiments, the at least one seed oil of a formulation of the invention may comprise, consist essentially of, or consist of soybean oil and coconut oil. In some embodiments, the at least one seed oil of a formulation of the invention may comprise, consist essentially of, or consist of cottonseed oil and coconut oil. In some embodiments, the at least one seed oil of a formulation of the invention may comprise, consist essentially of, or consist of soybean oil, cottonseed oil and coconut oil.

A formulation of the invention comprises at least one alcohol. Non-limiting examples of alcohols useful with the invention include ethanol, isopropyl alcohol, t-butyl alcohol, propyl alcohol, benzyl alcohol or any combination thereof. In some embodiments, the

alcohol may come from denatured alcohol and/or vodka. In some embodiments, the at least one alcohol of a formulation of the invention may comprise, consist essentially of, or consist of ethanol. In some embodiments, the at least one alcohol of a formulation of the invention may comprise, consist essentially of, or consist of denatured ethanol. In some embodiments, the at least one alcohol of a formulation of the invention may comprise, consist essentially of, or consist of isopropyl alcohol. In some embodiments, the at least one alcohol of a formulation of the invention may comprise, consist essentially of, or consist of t-butyl alcohol. In some embodiments, the at least one alcohol of a formulation of the invention may comprise, consist essentially of, or consist of propyl alcohol. In some embodiments, the at least one alcohol of a formulation of the invention may comprise, consist essentially of, or consist of benzyl alcohol. In some embodiments, the at least one alcohol of a formulation of the invention may comprise, consist essentially of, or consist of ethanol and isopropyl alcohol. In some embodiments, the at least one alcohol of a formulation of the invention may comprise, consist essentially of, or consist of ethanol, isopropyl alcohol and denatured alcohol.

Any denatured alcohol may be used in a formulation of the invention. In some embodiments, a "specially denatured alcohol" (SDA) may be used. In some embodiments, the denatured alcohol can be 95% ethanol and 5% isopropanol by volume. In some embodiments, the denatured alcohol can be 90% ethanol and 10% methanol by volume.

In some embodiments, a formulation of the invention comprises an equal amount of alcohol to hydrophobic base. In some embodiments, a formulation of the invention comprises about 3%-10% more alcohol than hydrophobic base (e.g., 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10% vol./vol. more alcohol than hydrophobic base) (e.g., about 40% alcohol to about 30% hydrophobic base, about 40% alcohol to about 35% hydrophobic base, about 38% alcohol to about 30% hydrophobic base, about 35% alcohol to about 32% hydrophobic base, about 35% alcohol to about 30% hydrophobic base, about 35% alcohol to about 25% hydrophobic base, about 30% alcohol to about 25% hydrophobic base, about 34% alcohol to about 30% hydrophobic base and the like). In some embodiments, a formulation of the invention comprises about two times the amount of hydrophobic base to alcohol (e.g., about 30% hydrophobic base to about 15% alcohol, about 40% hydrophobic base to about 20% alcohol, about 50% hydrophobic base to about 25% alcohol; about 60% hydrophobic base to about 30% alcohol, and the like).

A formulation of the invention further comprises emulsifiers to assist in maintaining a stable monophasic system. Non-limiting examples of an emulsifier include polysorbate 20,

sorbitan oleate, potassium sorbate, sodium benzoate, polysorbate 80, Tween 20, Tween 21, Tween 40, Tween 60, Tween 61, Tween 80, Tween 81, Tween 85; and/or sodium dodecyl sulfate (SDS). In some embodiments, at least one an emulsifier a formulation of the invention may comprise, consist essentially of, or consist of polysorbate 20, sorbitan oleate, potassium sorbate, and/or sodium benzoate. In some embodiments, the at least one emulsifier of the formulation may comprise, consist essentially of, or consist of polysorbate 20. In some embodiments, the at least one emulsifier of the formulation may comprise, consist essentially of, or consist of sorbitan oleate. In some embodiments, the at least one emulsifier of the formulation may comprise, consist essentially of, or consist of polysorbate 20 and sorbitan oleate.

In some embodiments, the formulations of the invention described herein may further comprise at least one of oil of lemon eucalyptus (OLE), p-methane-3,8-diol, and/or geraniol in an amount of about 5% to about 15% by volume (e.g., about 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, or any range or value therein; e.g., about 7% to about 12%, about 7% to about 13%, about 7% to about 14%, about 8% to about 11%, about 8% to about 12%, about 8% to about 13%, or any range or value therein). In some embodiments, the formulation may comprise geraniol in an amount of about 5% to about 15%. In some embodiments, the formulation may comprise OLE in an amount of about 5% to about 15%. In some embodiments, a formulation of the invention may comprise p-methane-3,8-diol in an amount of about 5% to about 15%. In some embodiments, a formulation of the invention may comprise geraniol and OLE in an amount of about 5% to about 15%. Thus, in exemplary embodiments, a formulation of the invention may comprise 2-undecanone in an amount of about 5% to about 50%, about 5% to about 30%, about 10% to about 20% or about 12% to about 18% by volume, or any range or value therein; at least one hydrophobic base in an amount of about 5% to about 65%, about 25% to about 40%, about 25% to about 35%, or about 27% to about 33% by volume, or any range or value therein; at least one alcohol in an amount of about 5% to about 50%, about 25% to about 40%, about 30% to about 40% or about 32% to about 38% by volume, or any range or value therein; at least one emulsifier in an amount of about 5% to about 15%, about 6% to about 13%, about 7% to about 12% or about 8% to about 12% by volume, or any range or value therein; and at least one of OLE, p-methane-3,8-diol, and/or geraniol in an amount of about 5% to about 15%, about 6% to about 13%, about 7% to about 12% or about 8% to about 12% by volume, or any range or value therein. In some embodiments, a formulation of the invention may comprise 2-undecanone in an amount of about 12% to about 18% by volume; at least one hydrophobic base in an

amount of about 28% to about 33% by volume, at least one alcohol in an amount of about 30% to about 35% by volume; at least one emulsifier in an amount of about 8% to about 12% by volume; and at least one of OLE, p-methane-3,8-diol, and/or geraniol in an amount of about 8% to about 12% by volume. In some embodiments, the formulations of the invention described herein comprise OLE in an amount of about 5% to about 15%, about 8% to about 12% or about 8% to about 13%, about 9% to about 12% by volume, and any range or value therein.

In some embodiments, a formulation of the invention may be anhydrous. In some embodiments, a formulation of the invention may comprise water in an amount less than 0.25% vol/vol. In some embodiments, a formulation of the invention may comprise water in a range from about 0.25% vol./vol. to about 50% vol./vol.

The formulations of the invention may be used to repel animals including, but not limited to, insects and ticks.

Accordingly, in some embodiments, the present invention provides a method of repelling an insect or a tick, comprising applying to a subject a formulation of the invention as describe herein in an amount effective to repel an insect or a tick. In some embodiments, an effective amount of a formulation of the invention can comprise by volume about 5% to about 50%, about 5% to about 40%, about 5% to about 30%, about 5% to about 20%, about 5% to about 17.5%, about 5% to about 15%, about 10% to about 50%, about 10% to about 40%, about 10% to about 30%, about 10% to about 20%, about 10% to about 17.5%, about 12% to about 17%, 13% to about 17%, 14% to about 16%, about 15% to about 50%, about 15% to about 40%, about 15% to about 30%, about 15% to about 20%, about 15% to about 17.5%, about 15% to about 18%, about 20% to about 50%, about 20% to about 40%, about 20% to about 30%, about 30% to about 50%, about 30% to about 40%, about 30% to about 50%, about 5%, about 6%, about 7%, about 8%, about 9%, about 10%, about 10.5%, about 11%, about 11.5%, about 12%, about 12.5%, about 13%, about 13.5%, about 14%, about 14.5%, about 15%, about 15.5%, about 16%, about 16.5%, about 17%, about 17.5%, about 18%, about 18.5%, about 19%, about 19.5%, about 20%, about 20.5%, about 21%, about 22%, about 23%, about 24%, about 25%, about 26%, about 27%, about 28%, about 29%, about 30%, about 35%, about 40%, about 45%, or about 50% of 2-undecanone, and any range or value therein.

In some embodiments, application of a formulation of the invention may be repeated as needed. Thus, in some embodiments, the application of the formulation may be repeated

every six hours, every 12 hours, every 24 hours, every other day, every three days, once a week, and the like, as needed.

In some embodiments, the formulations of the invention may be used to repel animals generally including, but not limited to, insects, ticks, spiders, hunting spiders, and/or scorpions. In some embodiments, the formulations of the invention may be used for repelling insects and ticks, wherein the insect may be, for example, a mosquito, sand fly, filth fly, chigger, flea, black fly, deer fly, horse fly, lice (e.g., body lice, head lice), roach, ant, fire ant, gnat, no-see um, and/or chigger, and the like.

The term "mosquito" as used herein refers to any type of mosquito (e.g., *Anopheles, Aedes,* and *Culex*), including but not limited to Tiger mosquitoes, *Aedes aboriginis, Aedes Aegypti, Aedes, albopictus, Aedes cantator, Aedes sierrensis, Aedes sollicitans, Aedes squamiger, Aedes sticticus, Aedes vexans, Anopheles quadrimaculatus, Culexpipiens,* and/or *Culex quinquefaxciatus.*

The term "tick" as used herein refer to any type of tick, including but not limited to, deer ticks, the American dog tick (*Dermacentor variabilis), Ornithodoros parkeri, O. moubata,* and/or *Dermacentor andersoni.*

The term "roach" as used herein refers to any type of roach, including but not limited to the American cockroach (*Periplaneta americana*), German cockroach *(Blattella germanica*), oriental cockroach (*Blatta orientalis),* wood cockroach (*Parcoblatta pennsylvanicd),* brownbanded cockroach (*Supella longipalpa),* and/or smokybrown cockroach *{Periplanetafuliginosd).*

In some embodiments, a formulation of the present invention may repel at least about 90% (e.g., at least about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 100%, and any value or range therein) (e.g., percent repellency) of the insects and/or ticks up to about seven hours (e.g., about 1 min, 5 min, 10 min, 15 min, 20 min, 25 min, 30 min, 35 min, 40 min, 45 min, 50 min, 55 min, 1 hr, 1.1 hrs, 1.2 hrs, 1.3 hrs, 1.4 hrs, 1.5 hrs, 1.6 hrs, 1.7 hrs, 1.8 hrs, 1.9 hrs, 2 hrs, 2.1 hrs, 2.2 hrs, 2.3 hrs, 2.4 hrs, 2.5 hrs, 2.6 hrs, 2.7 hrs, 2.8 hrs, 2.9 hrs, 3 hrs, 3.1 hrs, 3.2 hrs, 3.3 hrs, 3.4 hrs, 3.5 hrs, 3.6 hrs, 3.7 hrs, 3.8 hrs, 3.9 hrs, 4 hrs, 4.1 hrs, 4.2 hrs, 4.3 hrs, 4.4 hrs, 4.5 hrs, 4.6 hrs, 4.7 hrs, 4.8 hrs, 4.9 hrs, 5 hrs, 5.1 hrs, 5.2 hrs, 5.3 hrs, 5.4 hrs, 5.5 hrs, 5.6 hrs, 5.7 hrs, 5.8 hrs, 5.9 hrs, 6 hrs, 6.5 hrs, or 7 hrs or any range or value therein) after application of the formulation to a subject. Thus, in some embodiments, a formulation of the invention may be therapeutically effective (e.g., repels about 90% to 100% of insects and ticks) for about 4 hrs to about 7 hrs or more after application to a subject. In some embodiments, a formulation of the present invention may

repel at least about 90% to 100% of insects (e.g., mosquitoes) for up to about 4 hrs to about 6 hrs, about 4 hrs to about 5 hrs, about 5 hrs to about 6 hrs, or any range or value therein after application to a subject. In some embodiments, a formulation of the present invention may repel at least about 90% to 100% of ticks for up to about 5 hrs to about 7 hrs, about 5 hrs to about 8 hrs, about 6 hrs to about 7 hrs, about 6 hrs to about 8 hrs, or any range or value therein after application to a subject.

As is well understood, the repellency and duration of repellency for ticks versus insects such as mosquitoes can be different. This is due in part to the fact that odorant receptors on mosquitoes are on the antennae found on the head while those for ticks are on the tarsi (feet) on the front pair of legs. When repellency studies are conducted on mosquitoes, the mosquitoes are flying toward the human subject, detect the repellent that has evaporated from the skin of the human subject into the air some distance from the host (decreasing by 1 divided by distance squared from the repellent origin on the skin), and moving away from the repellent source (the skin). Because the mosquito is moving rapid through space in the direction of the host cues, the chemical repellency (amount of repellent in air and its repellent potency based on its unit of repellent activity per mole) must be high for repellency to occur. In the case of ticks, they are already on the host surface (skin or hair) or on host clothing and potentially moving toward a surface protected by the repellent. In this case, they are essentially walking on their olfactory surface, which comes into almost direct contact with the repellent treated surface. The repellent level in the air away from the host is not important but only that on or near the skin surface. The formulation developed in this invention was designed to provide the maximum duration of repellency for mosquitoes but also at same time provide maximum duration of repellency for ticks. Thus, in some embodiments, tick repellent duration may be longer because the tick detection and response to the repellent is at the repellent source on the skin surface and the duration of exposure to the repellent per unit time is higher since the tick is crawling at a much lower linear rate than mosquitoes that are flying toward the host. The formulation of the invention is designed to provide a longer tick duration than that for mosquitoes than current commercial repellents but at the same time provide a mosquito repellent duration competitive with current commercial mosquito repellents with comparative percentage levels of different active ingredients. Thus, in addition to insect and mosquito repellency, the present invention provides long lasting tick repellency that is advantageous in many locations where ticks may pose a greater human health risk than mosquitoes relative to the transmission of human diseases (for example, in the US and Europe).

In some embodiments, a formulation of the present invention may repel about 90% to about 100%, about 95% to about 98%, about 95% to about 99%, about 95% to about 100%, about 96% to about 98%, about 96% to about 99%, about 96% to about 100%, about 97% to about 98%, about 97% to about 99%, about 97% to about 100%, about 98% to about 99%, about 98 to about 100%, or about 99 to about 100% of the insects and/or ticks and any range or value therein for up to about seven hours after application of the formulation to a subject. In some embodiments, a formulation of the invention may repel about 95% to about 99% of the insects and ticks for up to about six hours after application of the formulation to a subject. In some embodiments, a formulation of the invention may repel about 95% to about 99% of the insects and ticks for up to about five hours after application of the formulation to a subject. In some embodiments, a formulation of the invention may repel about 98% to about 100% of insects and ticks for up to about four hours after application of the formulation to a subject. In some embodiments, a formulation of the invention may repel about 95 to about 99% of the insects and ticks for more than six hours (e.g., 6, 7, 8, 9, 10, 11, 12 hrs or more) after application of the formulation to a subject.

In some embodiments, a formulation of the invention may repel about 90% to about 100% (90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 100%, and any range or value therein) of insects and ticks for up to about 16 weeks (e.g., about 6 hours, 7 hours, 8 hours, 9 hours, 10 hours, 11 hour, 12 hours, 18 hours, 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 1 week, 2 weeks, 3 weeks, 4 weeks, 5 weeks, 6 weeks, 7 weeks, 8 weeks, 9 weeks, 10 weeks, 11 weeks, 12 weeks, 13 weeks, 14 weeks, 15 weeks, 16 weeks or more, or any range or value therein) when applied to a substrate. Thus, in some embodiments, a formulation of the invention applied to a substrate may repel about 90% to about 100%, about 95% to about 98%, about 95% to about 99%, about 95% to about 100%, about 96% to about 98%, about 96% to about 99%, about 96% to about 100%, about 97% to about 98%, about 97% to about 99%, about 97% to about 100%, about 98% to about 99%, about 98 to about 100%, or about 99 to about 100%, or any value or range therein, of insects and/or ticks for up to about 16 weeks and possibly longer after application of the formulation to the substrate.

In some embodiments, a determination of the percent of insects or ticks repelled by the formulation of the invention can be through the use of in an “arm in the cage” test or a “filter paper” test.

Filter yaper test. For the filter paper test, a lft xlft xlft Plexiglas with a stocking sleeve covering one end (the one end that does not contain a Plexiglass side) (e.g., a plastic cage with four sides and a bottom and a top and one side having a stocking sleeve instead of a

plastic side). The cage is filled with a sufficient number of mosquitoes (females only) so that when two grade number 1 filter paper discs (a control and a treated disc) (3.5 inches (9 cm) in diameter) are hung on the walls of the cage (on opposite sides of the cage), at least about 10 mosquitoes land per disc when the mosquitoes in the cage are excited into flight by blowing into the cage and then left undisturbed for 1 min. The assay is conducted at 25-27°C under low light conditions (humidity is not regulated but typical lab conditions). The environmental conditions are the same throughout the studies.

For each study, one filter paper disc is treated with the repellent (500 microliters per disc) and the other with the same solvent (500 microliters per disc) used to deliver the repellent. To evenly distribute the test compound and control solvent, the filter paper in each case is treated by application of small droplets added evenly over the entire surface of the paper. Filter paper is particularly useful because it allows the droplets to spread out from the point of application by absorption and diffusion within the cellulose disc. Once the filter paper discs are treated, they are laid horizontal on a glass surface on the lab bench at room temperature of about 25°C plus/minus 2°C and 60% relative humidity.

At different times after treatment of the paper, the discs are hung in the cage (the control (treated with carrier solvent only) is hung first and the disc carrying the formulations of the invention are hung second. The researcher handling the discs wears powder-free nitrile examination gloves. The process of placing the discs in the cage and also blowing into the cage by the researcher causes the mosquitoes to fly. Once the researcher removes his/her hands, the number of mosquitoes landing and remaining on the disc for more than three seconds is considered not to be repelled and is counted as such. The test is run over a fixed time, e.g., about 1 min). The researcher blows into the cage one additional time at the 30 sec interval into the 1 min observation period. The discs are removed in the same order that they were placed into the test cage by the researcher who is wearing a new pair of clean gloves. Since the discs are present in the cage for no more than about 1 min, any repellent released into the air will have minimum effects on the physiology of the mosquito. Percentage repellency is calculated as the number of mosquitoes landing on the control paper minus the number of mosquitoes landing on the treatment divided by the number of mosquitoes on the control paper times 100. Since the assay takes only 1 min to conduct, multiple compounds can be tested on the same cohort of mosquitoes. Periodically, the assay is checked to determine that no positional bias is occurring in the cage (e.g., the mosquitoes may favor one filter paper versus another due to the position of one of the filter paper discs relative to a light

source). Therefore, control experiments are conducted from time to time to determine if both filter paper discs untreated attract approximately the same number of mosquitoes.

Tick reyellencv filter paper test. Tick repellency was also determined using a filter paper test. In this case, naive, unfed, host-seeking (as determined by raised forelegs in response to the investigator's breath), mixed sex, adult ticks were used in all trials. Adult ticks (all three species) for use in repellency studies were held in plastic vials at about 28°C, about 75% relative humidity (RH), and a photoperiod of 14 hr light: 10 h dark, including dusk and dawn periods (60 min each). Behavioral bioassays were conducted at 25_C, 65% RH, and in complete darkness (except during the approximately 5 seconds to monitor tick distribution).

Ticks were allowed to choose between a treated and untreated filter paper surface. Tests were conducted in 63.6 cm^2 Petri plate lids lined with two 31.8 cm^2 semi-circle pieces of filter paper (Whatman no. 1), each treated with the formulations of the invention and allowed to dry for 3 h at room temperature under a fume hood. At 3 h, six ticks were placed in each arena along the line formed by the junction of treated and untreated papers. Distribution of ticks was recorded every 5 min from 5 to 30 min after the introduction of ticks to test arenas. An untreated control was used to determine the distribution of ticks in the absence of a repellent. Trials were repeated six times.

Arm-in-the case- test. For the arm in the cage test, the arm of a human subject (treated and untreated) is retained in the cage for 1 min.

A fixed amount of a test compound (e.g., formulation of the invention) (1 ml) is applied to one forearm by a researcher (the observer) who is wearing non-allergenic gloves on both hands. The observer applies the test compound using a pipette with a sterile tip and then spreads and massages the test compound into the skin from the elbow to the wrist on one arm of the subject (i.e., test arm).

At different times after application of the formulations of the invention, the subject applies a non-allergic glove to the hand on the test arm and inserts their arm into a cage of up to 200 female mosquitoes for about 1 min to about 5 min (e.g., about 1, 2, 3, 4, 5 min). A glove is used to cover the hand because the hand is not part of the assay - only the arm is used in the assay. The length of time may be varied depending on the activity of the mosquito or the mosquito species. It is known that different mosquito species and exact age of mosquito as well as unknown environmental and genetic variation in mosquitoes can affect activity (flying, landing and trying to bite). The repellency may be measured at multiple times post-treatment. Thus, the test may be run, for example, at about 4 hr, 5 hr, 6 hr, 7 hr, 8 hr, 9 hr, 10

hr, 11 hr, or 2 hr post-treatment. Between tests, the subject removes and disposes of the glove on the hand of the treated arm. For each time point, a new glove is used.

Mosquitoes that land on the skin for more than 2 seconds are counted as landing and the subject will move their arm in the cage so that the mosquito is not provided the opportunity to bite. An observer will count the number of landings.

The measurement of landings on the untreated arm (control arm) will precede the measurements made on treated arm. The subject will also have a glove on their untreated arm at each testing point, which will be replaced with a new glove for each time point.

The experiment is designed to observe about 200 or more landings on the untreated arm within one min. The mosquitoes are not allowed to take a blood meal and are prevented from doing so by shaking the arm. Any time a mosquito touches the arm, it is counted as a landing; that is, the mosquitoes do not have to land on the skin with their feet. The percentage repelled is determined by subtracting the number of insects, for example, mosquitos, that land on the treated arm from the number of mosquitos that land on the untreated arm and dividing this sum by the number of mosquitos that land on the untreated arm times 100.

The mosquitoes used in all tests are lab reared, disease free, female, and have not fed on blood of any type before the tests are conducted.

Thus, in some embodiments, a formulation of the present invention repels about 90% to about 100%, about 95% to about 98%, about 95% to about 99%, about 95% to about 100%, about 96% to about 98%, about 96% to about 99%, about 96% to about 100%, about 97% to about 98%, about 97% to about 99%, about 97% to about 100%, about 98% to about 99%, about 98 to about 100%, or about 99 to about 100% of the insects and/or ticks about four hours to about 7 hours after application of the formulation to an arm in the arm in the cage test, and any range or value therein. In some embodiments, a formulation of the invention repels about 100% of the insects and ticks about four hours after application of the formulation to an arm in an arm in the cage test. In some embodiments, a formulation of the invention repels about 99% of the insects and ticks about five hours after application of the formulation to an arm in the arm in the cage test.

Subjects to which the formulations of the present invention may be applied include human subjects and other animals (e.g., dogs, cats, horses, cattle). In some embodiments, a formulation is applied to a subject's skin and/or hair. In some embodiments, when the formulation of the invention is applied to a subject's hair the percent and time period of repellency may be greater than for application of the formulation to the subject's skin.

The formulations of the invention may be applied to subjects directly or indirectly, such as by applying the formulation to the skin or hair of the subject, or by applying the formulation to an article worn by or otherwise protecting/shielding the subject. As an example, a formulation of the invention may be applied to netting or screening that protects a subject.

A formulation of the invention may be applied to any substrate for which repelling animals, including insects and ticks is desired.

In some embodiments, a substrate may include, but is not limited to, fabrics (e.g., clothing, towels, linens, bed clothing, draperies, curtains, and the like); cotton products, wrist bands, wood products, furniture (e.g., chairs, mattresses, box springs, etc.), carpets, floors, soil and/or plants, containers, walls, base boards, air, soil, neting, paper, trash containers, dumps, tents, paint, etc.

Application to subjects or substrates may be carried out by any means that transfers the formulation to the subject or substrate, including but not limited to, spraying, misting, spreading, dusting, pouring, dipping, sprinkling, rubbing, fumigating, rolling, atomizing, and the like.

The formulations of the invention may be formulated in a variety of ways, including but not limited to liquids, powders, solids, emulsions, mousses, granules, dusts, a suspension, an aerosol (e.g., powdered, liquid), a shampoo, a conditioner, a creme rinse, a mousse, a foam, a lotion, a gel, a powder (e.g., a dry shampoo), a shampoo, a conditioner, a creme rinse, a paste, or an ointment. The formulations of the invention may be incorporated into or coated onto textiles, plastics, latex, rubber, cellulose, wicks, candles, paint, heat and air dispensers.

The invention will now be described with reference to the following examples. It should be appreciated that these examples are not intended to limit the scope of the claims to the invention, but are rather intended to be exemplary of certain embodiments. Any variations in the exemplified methods that occur to the skilled artisan are intended to fall within the scope of the invention.

EXAMPLES

Example 1. Procedures for testing repellent activity against the yellow fever mosquito *(Aedes aegypti)*, the Asian tiger mosquito (*Aedes albopictus)*, and *Anopheles gamhiae* (a malaria vector).

A fixed amount of repellent (1 ml) is applied to one forearm of the subject by a researcher (the observer) wearing non-allergenic gloves on both hands. The observer will apply the repellent using a Gilson pipetter with a sterile tip and then spread and massage the repellent into the skin using the pipette tip as a spreading device spreading the repellent from the elbow to the wrist on one arm of the subject (the test arm).

At different times after application of the repellent up to 12 h, the subject will apply a non-allergic glove to the hand on the test arm and then insert their arm into a cage of 200 female mosquitoes for 1-5 min. The length of time might be different depending on the activity of the mosquito or species; mosquito species and exact age of mosquito as well as unknown environmental and genetic variation in mosquitoes can affect activity (flying, landing and trying to bite). Post treatment measurements are made at 4, 5, 6, 7 and 8 h and possibly 12 h if repellency remains greater than 70% at 8 h. Between tests, the subjects remove and dispose of their glove on the hand of the treated arm. A new glove is used for each time point of testing.

Mosquitoes that land on the skin for more than 1 sec. will be considered ready to bite and the subject will move their arm in the cage so the mosquito leaves the skin to avoid actual biting of the subject. An observer will count the number of landings.

The subject has a glove on their untreated or control arm at each testing point. The insertion of the untreated arm will precede the treated arm and number of landings recorded as described. The glove is removed from the untreated arm immediately after removal from the cage as also will be the case for the treated arm. A new glove is used for each time point.

The study is stopped when percentage repellency is less than 50%. Percentage repellency is calculated based on the number of mosquitoes landing on the untreated arm minus the number landing on the treated arm divided by the number landing on the untreated arm times 100.

The mosquitoes used in the study are lab reared, disease free, adult female, and have not fed on blood of any type before the tests are conducted. Since the mosquitoes are not allowed to bite the subjects, there is also no chance for reactions to mosquito bites.

The mosquitoes are killed by freezing after the testing, autoclaved and disposed in the regular trash.

Example 2.

A combination of 2-undecanone with soybean oil and geraniol in the ranges tested provided an increased repellency over 2-undecanone in cotton seed soil and over 2-undecanone in soybean oil using polar solvents like water. In **Table 1,** the four component formulations were problematic for the following reasons: (i) the product separated into multiple phases when allowed to stand at room temperature; (ii) the product needed to be shaken to reform an emulsion before use (to re-establish a single phase) and as a result of (ii), the product (a) was predicted to have delivery issues as a commercial repellent possibly causing harm to users from the accidental application of neat ingredients and (b) would require specialized development of a new delivery system to apply the repellent because of the chemical degradation of the container parts by the separated 2-undecanone and/or geraniol (which adds to the cost of product development for the delivery system, potentially would result in development challenges that might not be solvable (including issues with having to shake the bottle before use) which is not consistent with industry standards for these type of products); further, the product (iii) was too viscous to be applied by aerosol; (iv) had a poor skin feel—felt oily and was uncomfortable to human test subjects; (v) had a high rate of contact transfer from skin to other materials like clothing that might affect product efficacy for mosquito and tick protection; (vi) did not achieve adequate mosquito repellency 100% at 5 h post treatment; (vii) had a strong, pungent smell which would not be desirable to users and hard to mask with other scents to make a final product with an acceptable odor; and (viii) produced undesirable skin reactions (all in part or whole from list given). Thus, the formulations in Table 1 demonstrated that alcohol could be used to decrease soybean oil content and therefore, improve feel on skin. However, these formulations achieved a repellency similar to that of the plant-based oil only.

Table 1. Results of repellency tests for selected formulations

Insect	*Aedes Aegypti*						
Formula Name	**Active ingredients**	**1hr**	**2hr**	**3hr**	**4hr**	**6hr**	**8hr**
Soybean 1	8% undecanone, 5% geraniol, 35% soybean oil, 52% isopropyl alcohol				*88.5% repellent		
Soybean 2	8% undecanone, 5% geraniol, 50% soybean oil, 37% isopropyl alcohol				*94.5% repellent		
Soybean 3	8% undecanone, 5% geraniol, 65% soybean oil, 22% isopropyl alcohol					*100% repellent	
Soybean 4	8% undecanone, 5% geraniol, 87% soybean oil					*100% repellent	
* 200 mosquitoes in the cage, calculation of percentage repellency based on number of landings on treated arm versus untreated arm (see example 1 for calculation details); undecanone=2-undecanone							

Example 3

Addition of alcohol breaks the heavy oily feel of the formulation and reduces the ease of transfer of the repellent from treated skin to items like clothing that might come into contact with treated skin. While, **Table 2** included a formulation with a different alcohol to plant-based oil concentration than those in **Table 1,** the negative aspects of the four component system discussed above in Example 2 were still present, including the separation of the formulation into different phases when left standing. Further, a higher level of plant-based oil was still needed to achieve the desired repellency; however, this higher amount of oil creates the undesirable greasy/oily feel discussed above. Thus, the combined data from **Tables 1-2,** helped establish the relationship between alcohol level and repellency in a dose dependent manner and assisted in moving closer to an alcohol/oil ratio that would provide 100% repellency and the best feel on the skin.

Table 2. Results of repellency tests for selected formulations

Insect	*Aedes aegypti*						
Formula Name	**Active, ingredients**	**1hr**	**2hr**	**3hr**	**4hr**	**6hr**	**8hr**
Soybean 5	8% undecanone, 5% geraniol, 25% soybean oil, 62% isopropyl alcohol				*73.5% repellent		
Soybean 1	8% undecanone, 5% geraniol, 35% soybean oil, 52% isopropyl alcohol				*88.5% repellent		
Soybean 2	8% undecanone, 5% geraniol, 50% soybean oil, 37% isopropyl alcohol				*94.5% repellent		
Soybean 3	8% undecanone, 5% geraniol, 65% soybean oil, 22% isopropyl alcohol					*100% repellent	
Soybean 4	8% undecanone, 5% geraniol, 87% soybean oil					*100% repellent	
* 200 mosquitoes in the cage, calculation of percentage repellency based on number of landings on treated arm versus untreated arm (see example 1 for calculation details); undecanone=2-undecanone.							

Example 4

The addition of specific emulsifiers prevented separation into multiple phases and improved the feel on the skin and the reduced transfer rates were maintained. However, the repellency of the formulations was reduced **(Table 3).** Also, the other negative characteristics (see Example 2, above) were still present. These data demonstrate the use of commercial grade denatured alcohol (ethanol) instead of isopropyl alcohol in the formulations. Based on dose response data in **Table 1** and **Table 2,** it is believed that the reduced repellency of the formulation was a result of reducing the percentage soybean oil.

Table 3. Results of repellency tests for selected formulations

Insect	*Aedes aegypti*			
Formula Name	**Active, ingredients**	**Control**	**4.0hr**	**% repellency**
Soybean 6	15% 2-undecanone, 5% polysorbate 20, 20% soybean oil, 55% denatured alcohol, 5% geraniol	205 landing/min	29 landing/min	*86%
Soybean 6	15% 2-undecanone, 5% polysorbate 20, 20% soybean oil, 55% denatured alcohol, 5% geraniol	240 landing/min	139 landing/min	*42%
* 200 mosquitoes in the cage, calculation of percentage repellency based on number of landings on treated arm versus untreated arm (see example 1 for calculation details).				

Example 5

Further experiments adjusting oil to alcohol ratio was needed to improve the desired repellency, maintain positive skin feel, to minimize accidental transfer from treated skin, to allow a stable monophasic system, and to eliminate issues associated with delivery of the repellent (issues as discussed in Example 2, above), see **Table 4.**

Table 4. Results of repellency tests for selected formulations

Insect	*Aedes aegypti*			
Formula Name	**Active, ingredients**	**Control**	**4.0hr**	**% repellency**
Soybean 7	15% 2-undecanone, 5% polysorbate 20, 30% soybean oil, 40% denatured alcohol, 5% geraniol, 5% sorbitan oleate	372 landing/min	23 landing/min	94%
Soybean 7	15% 2-undecanone, 5% polysorbate 20, 30% soybean oil, 40% denatured alcohol, 5% geraniol, 5% sorbitan oleate	292 landing/min	45 landing/min	85%
* 200 mosquitoes in the cage, calculation of percentage repellency based on number of landings on treated arm versus untreated arm (see example 1 for calculation details).				

Example 6

By further reducing the percentage of alcohol and adjusting other components, a desired repellency of over 95% was achieved in combination with a stable monophasic system, which addressed each of the issues raised in Example 2 (above).

The formulations of the invention are advantageous in that they do not easily rub off the skin and do not result in irritation to the skin when applied. Additionally, the formulations of this invention have an excellent feel and better activity than other known insect repellent formulations (e.g., BioUD, as shown in "arm in cage" studies). Thus, for example, as reported in Witting-Bissinger et al. (J. Med. Entomol., 45:891-898 (2008)) a commercial composition of 7.75% 2-undecanone (BioUD) had a mosquito repellency of about 70% at 5 hours post application as compared to 99% at 5 hours for the formulations of the present invention. Notably, in Witting-Bissinger et al., the cages contained 50 mosquitoes as compared to 200 in the cages used to test the formulations of the present invention. Thus, the formulations provided better repellency under greater biting pressure.

Example 7 Additional testing of the formulations of the invention

In this example, a formulation comprising 15% 2-undecanone, soybean oil, polysorbate 20, sorbitan oleate, oil of lemon eucalyptus and denatured alcohol was tested.

A colony of *Aedes aegypti* (L.) mosquitoes used in the bioassay was established from field-collected eggs from New Orleans, LA, in 2003. At 6 to 8 month intervals, adults reared from field-collected eggs were added to the mosquito colony to sustain genetic diversity. Larvae were reared as described by Trexler et al. (2003). Mosquito colonies were maintained in the Dearstyne Entomology laboratory at 28°C, 75% relative humidity, and a photoperiod of 14:10 (L:D), including two twilight periods (60 min each). Adult females used in the experiments were provided 10% sucrose solution *ad libitum* and were never allowed to blood feed.

The testing procedures were as follows. The test area was the surface of the arm from the elbow to the wrist (hands were covered with non-allergenic gloves). Test areas were washed with soap and water then swabbed with 70% ethanol before application of each repellent. The ethanol was allowed to evaporate completely before repellent treatments. Control arms were cleansed in the same manner. For all studies on human skin, repellents were applied at a rate of 1 ml/600 cm2. For each test, mated nulliparous host-seeking (non-blood fed) female mosquitoes aged between 6 and 18 d were released into a square 27,000

cm^3 Plexiglas cage with a sleeved opening on one side for insertion of a subject's arm. The cage contained only the test mosquitoes during the testing period (no nectar or water source).

For the arm in cage test, the arm of the human subject (treated versus untreated control) was inserted into the cage for 1 minute. Tests were conducted at 25°C, 60-70% relative humidity and under florescence laboratory lighting. A fixed amount of the test compound (1 ml) was applied to one forearm by a researcher (the observer) who was wearing non-allergenic gloves on both hands. The observer applied the test compound using a P1000 Pipetteman with a sterile tip and then spread (and massaged into the skin using the pipette tip) from the elbow to the wrist and one arm of the subject (i.e., the treatment arm). Prior to the study, the pipette calibration was validated by transferring distilled water to an analytical balance (+0.0001 grams) and was within the standard accuracy and precision of the manufacturer (<2% error). Nothing was applied to the other arm of the subject which was the control arm.

At different times after application of the formulation, the subject applied a non-allergic glove to the hand on the control and test arm and just afterward, inserted the arm into a cage (control arm first and then immediately the treatment arm); the cage contained up to 200 female mosquitoes. Each arm was inserted for 1 minute. The repellency was measured at multiple times post-treatment. Bioassays were conducted for each subject on the same cage of mosquitoes; different cages for different subjects. The time between bioassays was no shorter than 30 minutes and typically at least an hour. Insertion of the control versus treated arm did not cause a reduction in landing counts on the control arm at the different times tested. Between tests, the subject removed and disposed of the glove. For each time point, a new glove was used. The purpose of the glove was to prevent mosquito biting the hand, and no mosquito blood feeding on the hand was reported for any bioassay. The subject with a treated arm was encouraged to sit quietly and minimize physical activity.

Mosquitoes that touch the skin only are counted as landing; the mosquitoes do not have to stand on the skin with their legs to be considered a landing. When a landing was noticed where the mosquito is standing on the skin, the subject moved their arm in the cage in a rapid back and forth motion to prevent mosquito biting and blood feeding. No biting or blood feeding was noted in any of the bioassays. The observer counted the number of landings using a hand held numerical counter. The observer was positioned on the opposite side of the cage from the subject to best observe landings. The measurement of landings on the untreated arm (the control arm) preceded the measurements made on the treated arm. The

mosquito density was adjusted to achieve 200 or more landings on the untreated arm within one minute.

Percentage repellency provided was calculated as follows: (control landings treatment landings/control landings) *100.

The formulation tested in each of **Tables** 5-8, below, comprised 15% undecanone, soybean oil, polysorbate 20, sorbitan oleate, oil of lemon eucalyptus and denatured alcohol.

Table 5.

		At 4 hours		At 5 hours	
Subject	Control (land*/in)	Treatment (land/min)	% Repellency	Treatment (land/min)	% Repellency
1	320	19	94		
2	256	16	94		

*land = landings

Table 6.

		At 4 hours		At 5 hours	
Subject	Control (landVin)	Treatment (land/min)	% Repellency	Treatment (land/min)	% Repellency
1	312	1	99.7		
2	296	0	100		

*land = landings

Table 7.

Subject	Control (landVin)	At 4 hours Treatment (land/min)	% Repellency	At 5 hours Treatment (land/min)	% Repellency
1	156	2	98		
2	204	5	98		

*land = landings

Table 8.

Subject	Control (lancf/in)	At 4 hours Treatment (land/min)	% Repellency	At 5 hours Treatment (land/min)	% Repellency
1	216	3	99	2	99
2	468	3	99	4	99

*land = landings

The formulation of the invention was 99% effective for at least 5 hours in preventing mosquitoes even touching the subject's arm. In other trials with the formulation, the average repellency at 4 h was 95.3% (typical range 94 to 100%; Tables 5 to 7).

Example 8. The effect of the new formulations on the foraging behavior of ticks and mosquitoes.

In this example, a formulation comprising 2-undecanone, soybean oil, polysorbate 20, sorbitan oleate, oil of lemon eucalyptus and denatured alcohol was tested.

The objective of these laboratory studies was to quantify foraging behavior of mosquitoes and ticks in response to human study participants that were treated with the formulations of the invention.

In one study, mosquito landings were recorded on the untreated versus repellent-treated arms of human subjects. Exposures to mosquitos were made at 30-minute intervals, with each exposure lasting for 1 minute for treated arms and for 20 seconds for untreated control arms (to limit the number of bites received). Control exposures served to verify biting avidity of mosquitoes during the study. Complete Protection Time was defined as the time from application of the test material to a subject's skin until the exposure in which the first landing with intent to bite occurred that was followed by another landing with intent to bite within the same or the next exposure interval.

In a second study, crossings of individual ticks on untreated and repellent-treated arms of human subjects were recorded. Fresh ticks were first selected for avidity by initially checking that the ticks would traverse a specified distance on each subject's untreated arm within a three-minute period. "Qualifying" ticks were those that walked upward on an untreated arm, toward the elbow. Any tick that qualified was then moved to that subject's treated arm in order to challenge the repellent. Complete Protection Time was defined as the time from application until the exposure in which there was a crossing confirmed by another crossing within 30 minutes (First Confirmed Crossing). The earlier of these crossings was considered the end of Complete Protection.

Each study included four subjects. In the case of the study of the foraging behavior of mosquitoes, the negative control was the untreated arm of one subject. In the case of the study of the foraging behavior of ticks, the negative control was the untreated arm of each of the four subjects.

To minimize the chances of any potential substances (e.g., cosmetic products) on subject skin interfering with the test, each subject began by washing both arms with fragrance-free soap, rinsing with water, then washing both arms again with 70% ethyl alcohol in water, and finally rinsing with water. Arms were dried with plain paper towels prior to repellent applications. The topical repellent formulation of the invention was applied at a dosing rate of approximately of 1 mL per 600cm2 of skin surface area. Accordingly, the total amount of material applied to each subject was determined by the surface area of the forearms of each subject.

Mosquito testing.

Mosquito testing was against adult female *Aedes aegypti* from laboratory-reared disease-free populations.

The formulation tested in this example comprised 2-undecanone, soybean oil, polysorbate 20, sorbitan oleate, oil of lemon eucalyptus and denatured alcohol (e.g., "the test

material"). The test material was applied from the wrist to the elbow of each participant using a 1 mL syringe. Specifically, the repellent was distributed onto the skin in drops and lines, and then spread with two fingertips of one hand until complete and even coverage was observed. For each application, fresh gloves were used.

Approximately 100 laboratory-reared adult female mosquitoes were used to challenge the repellent in each exposure interval. Prior to testing, mosquitoes were supplied with feeding bottles of 10% sucrose in water. Feeding bottles were removed 12 hours prior to testing in order to increase their foraging avidity. Mosquitoes were held in 12-inch (~30 cm) cubic screened cages. During each exposure period, repellents were challenged in sequence in a single cage of mosquitoes. At 30-minute intervals beginning shortly after repellents were applied, each participant began exposures by inserting a forearm into a cage. For treated arms, exposures were for one minute, and for untreated control arms the exposure was for 20 seconds. Two observers counted mosquito landings during each exposure. Control exposures served to verify avidity in mosquitoes. A 20 second exposure of untreated arms to the mosquitoes was estimated to represent about 50% of the number that would be expected to land in a full minute exposure.

Repellent failure was scored independently for each subject, and defined as the time after application at which the first bite occurred that was followed by another bite within that or either of the next two intervals (time of First Confirmed Bite (FCB)).

Tick testins.

Tick tests used nymphal Deer Ticks, *Ixodes scapularis* from laboratory reared, disease-free populations and the formulation tested comprised 2-undecanone, soybean oil, polysorbate 20, sorbitan oleate, oil of lemon eucalyptus and denatured alcohol.

A single nymphal Deer Tick was used to challenge the repellent formulation of the invention during each exposure interval. Ticks were held in glass vials surrounded by water to contain them during the test, and fresh ticks were used for each challenge with a formulation of the invention. The formulation was challenged simultaneously in all subjects in 3 minute exposures commencing every 30 minutes beginning from the application of the test material.

Subjects manipulated ticks with small artist's paintbrushes, first placing them on an untreated arm to insure avid questing behavior in the absence of repellent, then placing them on the treated arm. In this way each subject served as their own negative control. Only avid ('qualified') ticks were used to challenge repellent formulations of the invention.

Crossings were measured by placing three dots on each subject's arms, 3-cm distant from one another, in a straight line (see, Fig. 1). For a treated arm, repellent was present above the middle dot, and failure was defined as crossing 3-cm into the treated area, past the third dot. An exposure interval where a tick did continue past this third dot was scored as a crossing, and repellent was considered no longer effective at that point if that crossing was confirmed by another within that or either of the next two intervals (First Confirmed Crossing, or FCC). For controls, the same crossing criterion was used to insure tick avidity, but no treatment (i.e., no repellent was present).

Repellent failure was scored independently for each subject, and defined as the time after application in which the first crossing occurred that was followed by another crossing in the next two intervals (First Confirmed Crossing).

Results

Mosquitoes

Of the four test participants, two received confirming landing with intent to bite, for Complete Protection Time (CPT) of 3 and 5 hours, respectively. The remaining two subjects withdrew voluntarily. One of the subjects that withdrew voluntarily received a single landing with intent to bite at 2 hours and withdrew voluntarily at 4.5 hours and the other received a single landing with intent to bite at 6 hours and then withdrew. With two subjects experiencing repellent failures and two withdrawing before failure, only a conservative mean CPT value can be calculated. Mean ± sd CPT was approximately 4.63 ± 1.25 hours.

While exposures were initiated at 30-minute intervals, they were generally 5-10 min past the exact 1/2 hour intervals post application. Hence each of the hour values below understates protection duration by about 5-10 minutes. Accounting for minor exposure delays in each period increases the conservative mean of approximately 4.75 hours. Accepting that the subjects who withdrew could not have received confirming landing with intent to bite until at least 30 minutes after their withdrawal times, the minimum reasonable mean CPT calculation is probably about 5.0 hours.

Ticks

None of the four subjects experienced any crossings on the treated arms. All subjects withdrew after several hours of testing confirmed to evident prolonged complete protection. Two subject withdrew after 6.5 hours, and the other two withdrew after 7 hours. The mean ± sd CPT against ticks was 6.75± 0.25.

Against ticks, no subjects tested their treatment all the way to failure. Thus, longer protection times could have been recorded had all subjects done so. Nonetheless, the average protection time among the data gathered was reasonably long and showed no signs of failure.

In conclusion, the topical repellent formulation of the invention was effective against *Aedes aegypti* mosquitoes for about 5 hours, and against *Ixodes scapularis* ticks for at least a few additional hours more than observed for the mosquitoes. We regard this duration of repellency against ticks to be strong.

Example 9. Animal Toxicity Tests

Any formulation for use as a repellant in addition to providing repellency at the same time has to be compatible to use on humans without adverse effects including not only skin but clothing, hair and accidental application to eyes and other sensitive surfaces on the body. It must also have no toxic effects and also have an acceptable relative to smell and feel on the skin and also relative to retention on treated surfaces with resistance to removal from typical rubbing that might occur to skin on different parts of the body. The formulations of the present invention were tested to show that they meet all of these criteria.

Test I. Skin Sensitization Study in Guinea Pigs

A formulation of the invention comprising 2-undecanone, soybean oil, polysorbate 20, sorbitan oleate, oil of lemon eucalyptus and denatured alcohol was tested for dermal sensitization potential utilizing a Buehler Technique Guinea Pig Sensitization Protocol. The test substance was evaluated for sensitization potential by applying 0.4 ml at a 100% concentration directly into Hilltop Chambers® and applying them to the clipped left shoulder of twenty albino guinea pigs in the following manner: The animals were held gently, and the chambers were applied as quickly as possible to the clipped left shoulder. The chambers were secured with Micropore tape and further secured with Kendall adhesive tape. Approximately six hours later, the tape and chambers were removed. Two additional induction doses were conducted following the same procedure, at weekly intervals.

Two weeks after the final application the animals received a topical primary challenge dose (6 hour contact) of the formulation at 100% concentration, on a naive site located on the right shoulder. Animals were scored for irritation at 24 and 48 hours after initiation of the primary challenge application.

Ten guinea pigs served as a naive control group, and remained untreated through the induction phase. Six naive control animals received only the primary challenge dose, at a

100% concentration. The four remaining guinea pigs were designated for a re-challenge, if necessary.

Following primary challenge with the formulation at 100% concentration, the incidence of grade 1 response or greater in the test group (0 of 20) was compared to that of the naive control group (0 of 6). The incidence and severity of these responses were not significantly greater than those produced by the naive control group indicating that sensitization had not been induced.

Test II. Acute Inhalation Toxicity Study in Rats

A formulation of the invention comprising 2-undecanone, soybean oil, polysorbate 20, sorbitan oleate, oil of lemon eucalyptus and denatured alcohol was tested for acute inhalation toxicity in accordance with OPPTS Guideline 870.1300 and OECD Guideline 403 at a targeted level of 2.0 mg/L of air by exposing five male and five female rats for a four hour period. The actual gravimetric concentration of the study was 2.33 mg/L of air. No mortality was observed during the 14 day observation period. The inhalation LC50 of the formulation was found to be greater than 2.33 mg/L of air for a 4 hour period. In accordance with the OPPTS Guidelines, formulation of the invention would be classified as EPA Toxicity Category IV for acute inhalation toxicity.

Test III. Acute Dermal Toxicity Study in Rabbits

A formulation of the invention comprising 2-undecanone, soybean oil, polysorbate 20, sorbitan oleate, oil of lemon eucalyptus and denatured alcohol was tested for acute dermal toxicity according to OPPTS, OECD Guidelines. The formulation, an amber liquid, was administered by dermal application at a dose of 5.0 g/kg body weight to five male and five female rabbits. No mortality occurred during the 14 day observation period. The acute dermal LD50 was found to be greater than 5.0 g/kg body weight. The formulation of the invention would be classified as Toxicity Category IV for dermal toxicity

Test IV. Acute Oral Toxicity Study in Rats

A formulation of the invention comprising 2-undecanone, soybean oil, polysorbate 20, sorbitan oleate, oil of lemon eucalyptus and denatured alcohol was tested for acute oral toxicity in accordance with OPPTS 870.1 100 and OECD 425 Guidelines. The formulation, an amber liquid, was administered by oral gavage at a 5000 mg/kg body weight limit dose level, according to the Agency's Up and Down Procedure to female rats. The acute oral

LD50 was determined to be greater than 5000 mg/kg body weight. Therefore, in accordance with OPPTS Guidelines, the formulation of the invention meets the requirements for EPA Toxicity Category IV for acute oral toxicity.

Test V Acute Dermal Irritation Study in Rabbits

A formulation of the invention comprising 2-undecanone, soybean oil, polysorbate 20, sorbitan oleate, oil of lemon eucalyptus and denatured alcohol was tested for primary dermal irritation in accordance with OPPTS and OECD Guidelines. The formulation, an amber liquid, was applied at a dose of 0.5 ml to an unabraded site on a clipped area of each of three albino rabbits. The application sites were graded for indication of skin reactions at 0.5, 24, 48, 72, 168 and 336 hours after test substance removal. There were mild skin irritation reactions in all of the test subjects which cleared by the 336 hour observation. The maximum skin irritation score was 3.0 at the 48 hour observation. The primary skin irritation index was calculated to be 2.58. In accordance with the OPPTS Guidelines, the formulation of the invention would be classified as EPA Toxicity Category IV for dermal effects.

Test VI. Acute Eye Irritation Study in Rabbits

A formulation of the invention comprising 2-undecanone, soybean oil, polysorbate 20, sorbitan oleate, oil of lemon eucalyptus and denatured alcohol was tested for eye irritation in accordance with OPPTS and OECD Guidelines. The formulation, an amber liquid, was administered into one eye of each of three albino rabbits. The eyes were observed and scored at 1, 24, 48, 72 and 168 hours. There was moderate eye irritation observed in all of the three test subjects which cleared by the 168 hour observation. The maximum group mean score was 14.67/1 10.0 at the 24 hour observation. In accordance with the OPPTS Guidelines, the formulation of the invention would be classified as Toxicity Category III.

Example 10. Human Repeat Insult Patch Tests for Skin Irritation and Skin Sensitization Evaluation

To determine the irritation and sensitization (contact allergy) potential of a test material after repeated application to the skin of human subjects three studies were conducted using the formulations of the invention comprising 2-undecanone, soybean oil, polysorbate 20, sorbitan oleate, oil of lemon eucalyptus and denatured alcohol (e.g., test materials).

Procedures:

Test materials to be tested under occlusive conditions were placed on an 8-millimeter aluminum Finn Chamber®(Epitest Ltd. Oy, Tuusula, Finland) supported on Scanpor® Tape (Norgesplaster A/S, Kristiansand, Norway) or an 8 mm filter paper coated aluminum Finn Chamber® AQUA supported on a thin flexible transparent polyurethane rectangular film coated on one side with a medical grade acrylic adhesive, consistent with adhesive used in state-of the-art hypoallergenic surgical tapes or a 7 mm IQ-ULTRA® closed cell system which is made of additive-free polyethylene plastic foam with a filter paper incorporated (It is supplied in units of 10 chambers on a hypoallergenic nonwoven adhesive tape; the width of the tape is 52 mm and the length is 118 mm) or other equivalents. Materials to be tested under semi-occlusive conditions were placed on a test strip with a Rayon/Polypropylene pad or on a 7.5 mm filter paper disc affixed to a strip of hypoallergenic tape (Johnson & Johnson 1 inch First Aid Cloth Tape).

Test materials to be tested in an open patch were applied and rubbed directly onto the back of the subject. Approximately 0.02-0.05 mL (in case of liquids) and/or 0.02-0.05 gm (in case of solids) of the test material was used for the studies. Liquid test material was dispensed on a 7.5mm paper disk, which fit in the Finn Chamber.

Subjects were requested to bathe or wash as usual before arrival at the facility. Patches containing the test material were then affixed directly to the skin of the intrascapular regions of the back, to the right or left of the midline and subjects were dismissed with instructions not to wet or expose the test area to direct sunlight. Patches remained in place for 48 hours after the first application. Subjects were instructed not to remove the patches prior to their 48 hour scheduled visit. Thereafter, subjects were instructed to remove patches 24 hours after application for the remainder of the study.

This procedure was repeated until a series of nine (9) consecutive, 24-hour exposures had been made three (3) times a week for three (3) consecutive weeks. Prior to each reapplication, the test sites were evaluated by trained laboratory personnel. Following a 10-14 day rest period a retest/challenge dose was applied once to a previously unexposed test site.

Test sites were evaluated by trained laboratory personnel 48 and 96 hours after application.

In the event of an adverse reaction, the area of erythema and edema were measured. Edema was estimated by the evaluation of the skin with respect to the contour of the unaffected normal skin. Subjects were instructed to report any delayed reactions that might occur after the final reading.

Scoring scale and definition of symbols shown below are based on the scoring scheme according to the International Contact Dermatitis Research Group scoring scale [Rietschel, R.L., Fowler, J.F., Ed., Fisher's Contact Dermatitis (fourth ed.). Baltimore, Williams & Wilkins, 1995] listed below:

0 no reaction (negative)
1 erythema throughout at least ¾ of patch area
2 erythema and induration throughout at least ¾ of patch area
3 erythema, induration and vesicles
4 erythema, induration and bullae
D Site discontinued
Dc Subject discontinued voluntarily
Del Subject discontinued per Investigator

Study I.

Formulation: 2-undecanone, soybean oil, polysorbate 20, sorbitan oleate, oil of lemon eucalyptus and denatured alcohol.

Population Demographics

Number of subjects enrolled	66
Number of subjects completing study	59
Age Range	19-65
Sex	
Male	4
Female	55
Fitzpatrick Skin Type*	
1 – always burn, does not tan	0
2 – burn easily, tan slightly	22
3 – burn moderately, tan progressively	8
4 – burn a little, always tan	17
5 – rarely burn, tan intensely	12
6 – never burn, tan very intensely	0

*Agache P., Hubert P.. *Measuring the skin*. Springer-Verlag **Berlin Heidelberg, 2004, (p. 473, table 48. 1)**

Results: No adverse reactions of any kind were reported during the course of this study. There were thirty-five (35) subjects with a Grade 1 reaction and four (4) subjects with a Grade 1 delayed reaction to the positive control (2.0% Sodium Lauryl Sulfate Solution).

No subjects showed any signs of reaction to the negative control (DI Water).

Study II.

Formulation: 2-undecanone, soybean oil, polysorbate 20, sorbitan oleate, oil of lemon eucalyptus and denatured alcohol.

Population Demographics:

Number of subjects enrolled	55
Number of subjects completing study	55
Age Range	18-64
Sex	
Male	9
Female	46
Fitzpatrick Skin Type*	
1 – always burn, does not tan	0
2 – burn easily, tan slightly	0
3 – burn moderately, tan	55
4 – burn a little, always tan	0
5 – rarely burn, tan intensely	0
6 – never burn, tan very intensely	0

*Agache P., Hubert P.. *Measuring the skin.* Springer-Verlag Berlin Heidelberg, 2004, (p. 473, table 48.1)

Results : No adverse reactions of any kind were reported during the course of this study. There were Ten (10) subjects with a Grade 1 reaction to the positive control (2.0% Sodium Lauryl Sulfate Solution). No subjects showed any signs of reaction to the negative control (DI Water).

Study III

Formulation: 2-undecanone, soybean oil, polysorbate 20, sorbitan oleate, oil of lemon eucalyptus and denatured alcohol.

Population Demographics:

Number of subjects enrolled	55
Number of subjects completing study	54
Age Range	19-63

Sex	
Male	5
Female	49
Fitzpatrick Skin Type*	
1 – always burn, does not tan	0
2 – burn easily, tan slightly	0
3 – burn moderately, tan progressively	54
4 – burn a little, always tan	0
5 – rarely burn, tan intensely	0
6 – never burn, tan very intensely	0

*Agache P., Hubert P.. *Measuring the skin.* Springer-Verlag Berlin Heidelberg, 2004, (p. 473, table 48.1)

Results : No adverse reactions of any kind were reported during the course of this study. Two subjects showed Grade 1 and two subjects showed Grade 2 reaction to the positive control (2.0% Sodium Lauryl Sulfate Solution). No subjects showed any signs of reaction to the negative control (DI Water).

The foregoing is illustrative of the present invention, and is not to be construed as limiting thereof. The invention is defined by the following claims, with equivalents of the claims to be included therein.

THAT WHICH IS CLAIMED IS:

1. A formulation comprising:

2-undecanone in an amount of about 5% to about 50% by volume in the formulation;

at least one hydrophobic base in an amount of about 5% to about 65% by volume in the formulation;

at least one alcohol in an amount of about 5% to about 50% by volume in the formulation; and

at least one emulsifier in an amount of about 5% to about 15% by volume in the formulation.

2. A formulation comprising:

2-undecanone in an amount of about 5% to about 30% by volume in the formulation;

at least one hydrophobic base in an amount of about 25% to about 40% by volume in the formulation;

at least one alcohol in an amount of about 25% to about 40% by volume in the formulation; and

at least one emulsifier in an amount of about 5% to about 15% by volume in the formulation.

3. A formulation comprising:

2-undecanone that repels insects and ticks in an amount of about 10% to about 25% by volume in the formulation;

at least one hydrophobic base in an amount of about 25% to about 35% by volume in the formulation;

at least one alcohol in an amount of about 25% to about 40% by volume in the formulation; and

at least one emulsifier in an amount of about 5% to about 15% by volume in the formulation.

4. The formulation of any one of claims 1 to 3, further comprising at least one of oil of lemon eucalyptus (OLE), p-menthane-3,8-diol, and/or geraniol.

5. The formulation of any one of claims 1 to 4, wherein the at least one hydrophobic base is a branched hydrocarbon, an unbranched hydrocarbon, a hydrophobic polymer, a fatty acid, a fatty acid ester, a monoglyceride, a diglyceride, and/or a triglyceride.

6. The formulation of any one of claims 1 to 5, wherein the at least one hydrophobic base is a plant-based oil.

7. The formulation of claim 6, wherein the plant-based oil is avocado oil, borage oil, canola (rapeseed) oil, castor oil, chamomile oil, coconut oil, corn oil, cottonseed oil, rapeseed oil, evening primrose oil, safflower oil, sunflower oil, soybean oil, sweet almond, palm oil, palm kernel oil, arctium lappa seed oil, sesame oil, borgo officialis seed oil, brassica campestris oleifera oil, brevoortia oil, bubulum oil, cistus ladaniferus oil, elaeis guineensis oil, almond oil, pine oil, olive oil, peanut oil, flax seed oil, wheat germ oil, hemp oil, jojoba oil, linseed oil, macadamia oil, neem oil, argan oil, grape seed oil, thistle oil, and/or algal oil and/or any combination thereof.

8. The formulation of claim 6, wherein the plant-based oil is soybean oil, cottonseed oil and/or coconut oil.

9. The formulation of any one of claims 1 to 8, wherein the at least one alcohol is ethanol, isopropyl alcohol, t-butyl alcohol, propyl alcohol, and/or benzyl alcohol.

10. The formulation of any one of claims 1 to 8, wherein the at least one alcohol is ethanol, isopropyl alcohol and/or denatured alcohol.

11. The formulation of any one of claims 1 to 10, wherein the at least one emulsifier is polysorbate 20, sorbitan oleate, potassium sorbate, and/or sodium benzoate.

12. The formulation of any one of claims 1 to 11, wherein the formulation comprises an equal amount of the at least one alcohol to the at least one hydrophobic base.

13. The formulation of any one of claims 1 to 12, wherein the formulation comprises the at least one hydrophobic base in an amount that is about two times greater than the amount of the at least one alcohol.

14. The formulation of any one of claims 1 to 13, wherein the formulation comprises the at least one alcohol in an amount that is about 3% to about 10% (vol./vol.) greater than the amount of the at least one hydrophobic base.

15. The formulation of any one of claims 1 to 14, wherein formulation is a monophasic or single phase composition (e.g., a monophasic or single phase solution).

16. A method of repelling an insect pest or a tick, comprising:

applying the formulation of any one of claims 1-15 to a subject or a substrate in an amount effective to repel the insect or tick.

17. The method of claim 16, wherein the insect is a mosquito, sand fly, filth fly, chigger, flea, black fly, deer fly, horse fly, lice (e.g., body lice, head lice), roach, ant, and/or fire ant.

18. The method of claim 16 or claim 17, wherein about 90% to about 100% of the insects and/or ticks are repelled.

19. The method of claim 16 or claim 17, wherein about 95% to about 100% of the insects and/or ticks are repelled.

20. The method of claim 16 or claim 17, wherein about 98% to about 100% of the insects and/or ticks are repelled.

21. The method of any one of claims 18 to 20, wherein the insects and/or ticks are repelled for up to about 7 hours after application.

22. The method of any one of claims 16 to 21 wherein the subject is a human.

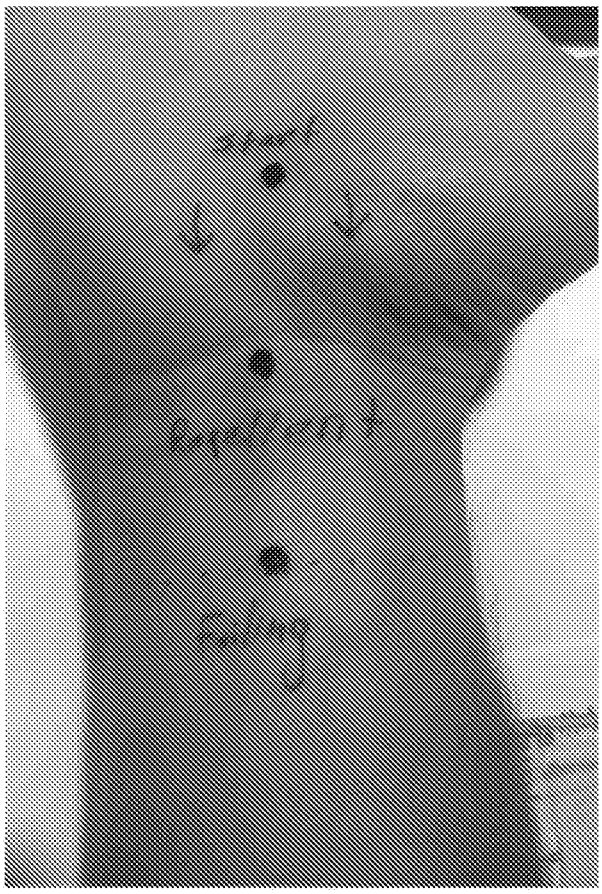
23. The method of any one of claims 16 to 22, wherein the substrate is fabric, cotton products, clothing, wrist bands, bedding, linens, towels, curtains, draperies, wood products, furniture, carpet, floors, walls, baseboards, netting, paper, water, trash containers, dumps, tents, paint, plants, and/or soil.

24. A product infused with the formulation of any one of claims 1 to 15.

25. The product of claim 24, wherein the product is a textile, a plastic, latex, rubber, cellulose, a wick, a candle, paint, a cosmetic, a lotion, a shampoo, a conditioner, and/or a mousse.

26. The product of claim 25, wherein the textile is clothing, bedding, linens, towels, curtains, draperies, tents, and/or netting.

Fig. 1



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 18/67625

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A01N 31/02, A01N 33/08, A01N 35/02 (2019.01)
CPC - A01N 25/00, A01N 31/04, A01N 31/16

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
See Search History Document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
See Search History Document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
See Search History Document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2010/0227010 A1 (Jones) 09 September 2010 (09.09.2010); entire document, especially abstract, [0034], [0077], [0079], [0081]	1-4
A	US 2004/0242703 A1 (Roe) 24 December 2004 (24.12.2004); entire document	1-4
A	CN 106847691 A (Hu Xuejun) 13 June 2017 (13.06.2017); entire document	1-4
X, P	WO 2018/026901 A1 (NORTH CAROLINA STATE UNIVERSITY) 08 February 2018 (08.02.2018); entire document	1-4

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
05 March 2019	26 MAR 2019

Name and mailing address of the ISA/US	Authorized officer:
Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 18/67625

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 5-26
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

insects

MDPI

Article

Imergard^{TM}WP: A Non-Chemical Alternative for an Indoor Residual Spray, Effective against Pyrethroid-Resistant *Anopheles gambiae* (s.l.) in Africa

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Abstract: Malaria is the deadliest mosquito-borne disease and kills predominantly people in sub-Saharan Africa (SSA). The now widespread mosquito resistance to pyrethroids, with rapidly growing resistance to other insecticide classes recommended by the World Health Organization (WHO), may overturn the successes gained in mosquito control in recent years. It is of utmost importance to search for new, inexpensive, and safe alternatives, with new modes of action, that might improve the efficacy of current insecticides. The efficacy of a novel mechanical insecticidal mineral derived from volcanic rock, Imergard^{TM}WP, was investigated to determine its efficacy as a stand-alone residual wall spray and as a mixture with deltamethrin (K-Othrine® Polyzone) in experimental huts in Cove, Benin. The evaluation was conducted with susceptible (Kisumu) and wild-type *Anopheles gambiae* (s.l.). Deltamethrin applied alone demonstrated 40–45% mortality (at 72 h post-exposure) during the first four months, which declined to 25% at six months for wild *An. gambiae* from Cove. Imergard^{TM}WP alone and mixed with deltamethrin, under the same assay conditions, produced 79–82% and 73–81% mortality, respectively, during the same six-month period. Imergard^{TM}WP met the 80% WHO bio-efficacy threshold for residual activity for the first five months with 78% residual activity at six months. Imergard^{TM}WP can be used as a mixture with chemical insecticides or as a stand-alone pesticide for mosquito control in Africa.

Keywords: mosquito; *Anopheles gambiae* (s.l.); malaria; Imergard^{TM}WP; mechanical insecticide; pyrethroid resistance; Africa; Benin; residual wall spray

1. Introduction

Malaria continues to be a major life-threatening vector-borne disease globally but more so in developing countries. According to the World Health Organization (WHO), over 90% of the 228 million

malaria cases and 405,000 deaths observed in 2018 were recorded in Africa [1]. It is the first cause of mortality and morbidity in West Africa, and the majority of malaria-endemic countries are in sub-Saharan Africa (SSA), which shares 80% of the global malaria burden [1,2]. Malaria parasites are transmitted by infected Anopheline mosquito species. Vector control is therefore essential in the fight against malaria and is commonly accomplished through the use of insecticide-treated nets (ITNs) and indoor residual spraying (IRS) [3]. The latter consists of applying insecticides to interior walls of a house.

IRS is highly effective in controlling malaria vectors and has contributed significantly to the successes obtained in the fight against the disease in recent years [4,5]. However, insecticide resistance is now widespread for pyrethroids, one the most used insecticide classes in vector control [6–8]. More concerning is that resistance to the other classes of insecticides approved by the WHO (carbamates, organochlorines, and organophosphates) has started to rise and has now been suspected or documented in several countries worldwide [6,9–11]. It is therefore of utmost importance to search for alternatives that not only exhibit new modes of action, but are also safer, cost-effective, sustainable, naturally occurring if possible, and compatible with the synanthropic nature of mosquitoes.

Mechanical insecticides (MIs) are minerals that come into contact with insects and produce a lethal effect. MIs exhibit insecticidal properties through a physical mode of action and have consistently been shown to control numerous agricultural pests, i.e., Coleoptera [12], Thysanoptera [13], Lepidoptera [14], and Hemiptera [15]. Their potential use in public heath, on the other hand, has received minimal attention and was almost exclusively limited to laboratory trials [16,17].

In this study, we demonstrated the efficacy of a new mineral-based mosquitocide derived from volcanic rock, Imergard^{TM}WP (expanded perlite, 100%), in commercialization by the company, Imerys. Perlite is an aluminosilicate volcanic glass with a relatively high-water content, typically formed by the hydration of obsidian. It occurs naturally and has the unusual property of greatly expanding in volume under extreme temperature. Because of its low density, perlite is used as insulation or in plant growth media. Perlite is listed in the US Food and Drug Administration (FDA) database as Generally Recognized as Safe (GRAS), is classified as a feed additive by the European Food Safety Authority (EFSA), and is found in products like toothpaste [18]. It has not been considered before for vector control. The MI described in this paper (applied by spraying in water) provides a new mode of insecticide action for mosquito and malaria control, disrupting insect water balance. The current thinking is that mechanical insecticides disrupt the protective lipid layer of the insect's cuticle, thus leading to death by desiccation (a non-toxic, i.e., non-systemic mode of action) [16,17,19]. Because the MI is an industrial mineral, its activity does not degrade with time as long as the mechanical insecticide is present and can transfer to the insect [19]. Since mechanical insecticides have not been used before in mosquito control, it should be effective against pyrethroid-resistant mosquitoes in Africa.

We evaluated the toxicity and residual activity of Imergard^{TM}WP alone and as a mixture with deltamethrin for the control of susceptible and wild-type pyrethroid-resistant mosquitoes under field conditions in Africa. We demonstrated that the MI is a suitable alternative to a chemical insecticide currently in use for mosquito control and offers a long-lasting protection against mosquitoes such as the African malaria mosquito, *Anopheles gambiae* (s.l.).

2. Materials and Methods

2.1. Study Site and Experimental Huts

The study was conducted in the experimental hut station of Cove, southern Benin (7°14′ N, 2°18′ E) for six months (July to December 2017). The rice-growing fields surrounding the station provide year-round and prolific breeding sites for *An. gambiae* (s.l.). The local *Anopheles* populations are highly resistant to pyrethroids as shown from studies conducted in 2013–2014 [20] and confirmed in 2015 [21,22]. Eight experimental huts of WHO-approved West African design were used [23]. The huts were concrete experimental huts with corrugated iron roofs. The ceilings were polyethylene sheeting

covered with palm thatch, and the interior walls were plastered with cement. Four window slits (1 cm gap, 2 on the front, 2 on the sides) allowed entrance of host-seeking mosquitoes but impeded their exit, forcing them to fly towards the veranda trap on the back of the hut. A schematic of the hut can be found in Hougard et al. [24].

2.2. Insecticide Treatments

Four treatments were evaluated, each randomly applied (2 huts per treatment, 8 huts in total) as follows: (i) unsprayed, control huts, (ii) deltamethrin 62.5 SC-PE (K-Othrine® Polyzone; Bayer, Monheim am Rhein, Germany) at 25 mg/m^2, (iii) Imergard^{TM}WP (also referred to as ImG in this paper) (Imerys, Roswell, GA, USA) at 8 g/m^2 as a wettable powder, and (iv) deltamethrin 62.5 SC-PE at 25 mg/m^2 + Imergard^{TM}WP at 4 g/m^2. The MI was applied as a wettable powder per manufacturer's recommendations. Maximum safety instructions and protective measures were observed as a standard practice for all treatments [22]. The huts (walls and ceiling) were sprayed using a 10L Hudson Xpert compression sprayer (Hudson Xpert, Chicago, IL, USA). A mixture was conducted to test potential potentiation between Imergard^{TM}WP and a chemical insecticide.

2.3. Sleepers and Mosquito Collection

Eight consenting human (adult) volunteers were locally recruited and served as sleepers (mosquito collectors). They were rotated between huts on successive nights to simulate a Latin square design. They slept in the huts from 21:00 to 6:00 each night. Each morning (from 6:00), mosquitoes were collected from the huts using a mouth aspirator (main room and veranda) and transferred to the laboratory where they were identified using appropriate identification keys [25]. They were also scored as dead or alive and as fed or unfed. Mosquitoes were scored as dead if they did not move after being touched with a blunt probe. Mosquitoes were considered fed if the abdomen was visually engorged with blood. Live mosquitoes were kept in small cups, and delayed mortality was recorded for up to 72 h for all four treatments. Because the putative mode of action of the MI is desiccation (as mentioned earlier) and preliminary studies suggested that providing water sources in large cage tests had no impact on mortality, mosquitoes were not provided with a sugar solution during the holding period.

The data from the two huts for each treatment were pooled together, and treatment efficacy was expressed relative to the untreated control in terms of deterrence (reduction in hut entry relative to the untreated control huts), the exophily rate (proportion of mosquitoes, relative to that of the control, that were found in the exit trap (= veranda)), blood-feeding inhibition (reduction in blood feeding compared with that of the control), and delayed mortality (percentage mortality at 24, 48, and 72 h post-collection).

2.4. Residual Activity of Insecticide Treatments

The residual activity of the different insecticide treatments was determined one week after treatment application and for each month of the trial using WHO cone bioassays [23]. Laboratory maintained susceptible female *An. gambiae* "Kisumu" and wild *An. gambiae* (s.l.) from Cove were tested. Ten mosquitoes (non-blood fed, 2–5 days old with *ad libitum* access to a 10% honey-in-water solution before the transfer to cones) were introduced per cone and exposed to the walls for 30 min at different heights from the hut floor (0.5, 1, 1.5, and 2 m). Mortality was then recorded at 24 and 48 h.

2.5. Statistical Analyses

The raw data were managed using Microsoft Excel (Version 2016, Redmond, WA, USA). The WHO bio-efficacy threshold was used for the analyses of the residual effect [23]. Statistical analyses on deterrence, exophily, and blood feeding between the control and treatment were conducted using a two-proportion *Z*-test. The 95% confidence intervals for the proportions were computed using the binomial test. Control mortality for mosquitoes entering the huts ranged from 2.4% to 4.5% at 72 h at each time point over six months. The control residual activity for the Kisumu strain in cone tests

was 4.2–16% at 48 h over six months with the majority of observations <5%. The control residual activity of the wild population was 1.6–15% at 48 h with the majority <5%. Mortality rates were Abbott corrected [26]. All analyses were completed using the R statistical software (Version 3.6.1, R Foundation for Statistical Computing, Vienna, Austria) (R Development Core Team 2016) [27].

2.6. Ethical Clearance

The study was approved by the Ministry of Health in Benin (Comité d'Ethique Institutionnelle du CREC, Avis éthique favorable No. 03 du 07 Juillet 2017, Lettre No 231/MS/DC/SGM/DRFMT/CREC/CEI-CREC/SA du 03 Août 2017). The human volunteer sleepers gave informed consent prior to their participation in the study.

2.7. Abbreviations

CREC: Centre de Recherche Entomologique de Cotonou; Delta: deltamethrin; DM, deltamethrin; EFSA: European Food Safety Authority; FDA: Food and Drug Administration; GRAS: generally recognized as safe; ImG: Imergard^{TM}WP; IRS: indoor residual spraying; ITNs: insecticide-treated nets; MI: mechanical insecticide; NCSU: North Carolina State University; PE: polymer enhanced; SEM: scanning electron microscopy; SC: suspension concentrate; SE: standard error; WG: water dispersible granules; WHO: World Health Organization; WP: wettable powder.

2.8. Data Availabity

All of the data supporting the findings are presented in the paper.

3. Results

The efficacy of ImG (Figure 1) was evaluated in small-scale field trials in southern Benin, West Africa (Figure 2A). The MI readily mixed with water with minimal agitation and remained in suspension long enough for conducting the applications without additional agitation. Standard vector control sprayers (as described in the Materials and Methods section) without modification were adequate for the application, and Figure 2B shows the MI applied at the rate of 8 g per square meter to interior hut walls and the ceiling. The product was odorless and, once applied to the wall, did not produce any obvious airborne dust. The studies were conducted using WHO-approved standard research huts (Figure 2C,D) following WHO protocols [23]. The parameters tested are presented below.

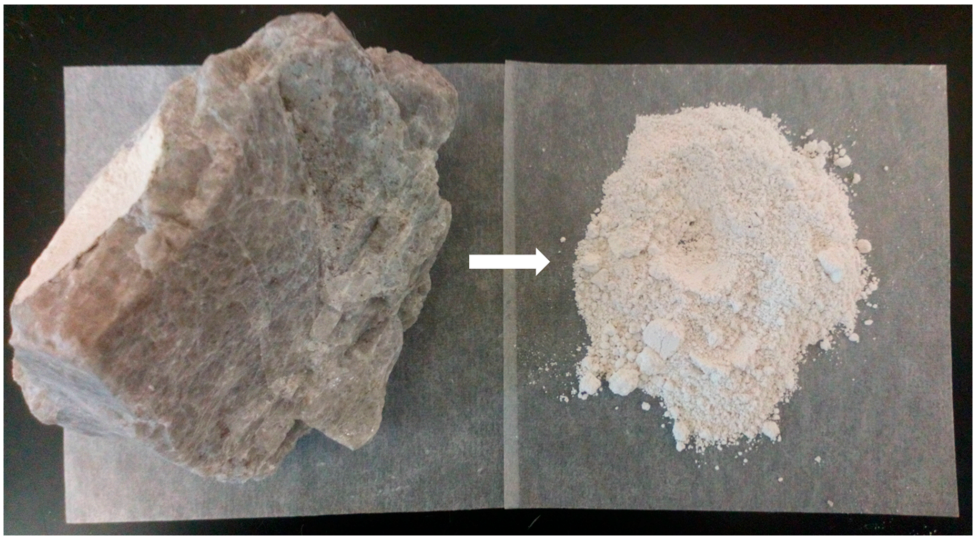


Figure 1. Imergard^{TM}WP before and after processing. Perlite, the active ingredient of Imergard^{TM}WP, is a volcanic rock (**left**) that can be expanded (when heated to 760–980 °C) and then processed (drying and grinding) to obtain the fine white powder used in this study (**right**).

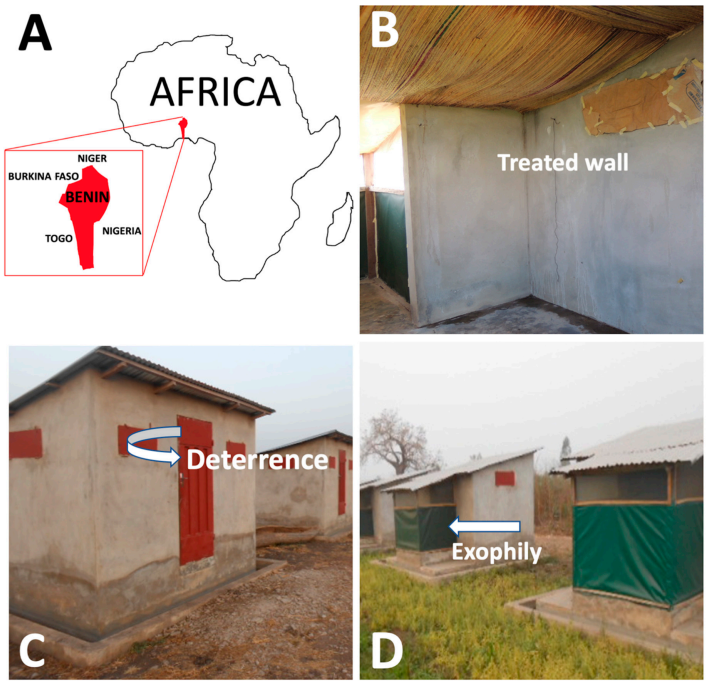


Figure 2. Study site and experimental huts: (**A**) location of field trial in Africa; (**B**) appearance of a treated wall; (**C**) front and side views of an experimental hut displaying the window slits for mosquito entrance as well as the movement of deterred mosquitoes; and (**D**) back view of a hut displaying the veranda trap as well as the exophily movement of mosquitoes that had entered the hut.

3.1. Deterrence

In total, 26,551 host-seeking *An. gambiae* (s.l.) were collected during the six-month trial. In general, and compared to the untreated control, all the treatments reduced mosquito entry at rates ranging between 8% and 39% (Table 1). However, this effect (Figure 2C) was highly significant ($p < 0.0001$) and more noticeable in the huts sprayed with a mixture of ImG and deltamethrin 62.5 SC-PE where it lasted for six months (Table 1).

Table 1. Hut entry reduction in wild-type *Anopheles gambiae* (s.l.).

Treatment	Months	Total [a]	Proportion (%) [b]	95% CI [c]	*p*-Value [d]
Control	1	785			
	2	331			
	3	592			
	4	796			
	5	2084			
	6	2355			
Deltamethrin	1	716	8.8	6.7–10.9	<0.0001
	2	203	38.7	33.1–44.2	<0.0001
	3	542	8.4	6.0–10.8	<0.0001
	4	838	-	-	>0.05
	5	1918	8.0	6.8–9.2	<0.0001
	6	2539	-	-	>0.05
Imergard^{TM}WP	1	645	17.8	15.0–20.6	<0.0001
	2	204	38.4	32.8–43.9	<0.0001
	3	556	6.1	4.0–8.2	<0.0001
	4	719	9.7	7.5–11.8	<0.0001
	5	2098	-	-	>0.05
	6	2658	-	-	>0.05

Table 1. *Cont.*

Treatment	Months	Total [a]	Proportion (%) [b]	95% CI [c]	*p*-Value [d]
DeltaM + ImG [e]	1	587	25.2	22.1–28.4	<0.0001
	2	257	22.4	17.6–27.2	<0.0001
	3	527	11.0	8.3–13.7	<0.0001
	4	678	14.8	12.2–17.4	<0.0001
	5	1760	15.6	13.9–17.2	<0.0001
	6	2163	8.2	7.0–9.3	<0.0001

[a] Total number of mosquitoes collected in huts for each month indicated. [b] Percentage reduction of mosquitoes found in treated huts compared to the number of mosquitoes found in the control; dashes represent values less than zero. [c] CI, confidence interval; dashes indicate no CI was calculated. [d] Two-proportion *Z*-test, $\alpha = 0.05$. [e] Deltamethrin and Imergard^{TM}WP mixed together and then sprayed.

3.2. Exophily

All treatments elicited an increased exit behavior from the mosquitoes (Figure 2D, Table 2). On average, the proportion exiting the control huts was 31%. Exophily rates with deltamethrin 62.5 SC-PE (56% on average) and the mixture of ImG with deltamethrin 62.5 SC-PE (50%) were significantly ($p < 0.0001$) higher than the control huts. Exophily rates did not differ significantly between ImG (33%) and the control (31%) ($p > 0.05$).

Table 2. Exophily rates [a] at different times after spraying for wild-type *An. gambiae* (s.l.).

Treatment	Months	Total [b]	Proportion (%) [a]	95% CI [c]	*p*-Value [d]
Control	1	785	26.9	23.8–30.1	-
	2	331	26.3	21.6–31.4	-
	3	592	31.6	27.9–35.5	-
	4	796	30.2	33.5–37.0	-
	5	2084	34.7	32.6–36.8	-
	6	2355	33.8	31.9–35.8	-
Deltamethrin	1	716	53.2	49.5–56.9	<0.0001
	2	203	56.2	49.0–63.1	<0.0001
	3	542	53.7	49.4–58.0	<0.0001
	4	838	56.4	53.0–59.8	<0.0001
	5	1918	59.8	57.6–62.0	<0.0001
	6	2539	57.1	55.1–59.0	<0.0001
Imergard	1	645	31.0	27.4–34.7	0.097
	2	204	34.8	28.1–41.6	0.045
	3	556	31.3	27.5–35.3	0.966
	4	719	33.2	29.8–36.8	0.216
	5	2098	33.6	31.5–35.6	0.458
	6	2658	33.5	31.7–35.3	0.858
DeltaM+ ImG [e]	1	587	48.2	44.1–52.3	<0.0001
	2	257	48.6	42.4–54.9	<0.0001
	3	527	48.4	44.0–52.8	<0.0001
	4	678	48.7	44.8–52.5	<0.0001
	5	1760	49.9	47.2–52.0	<0.0001
	6	2163	50.2	48.0–52.3	<0.0001

[a] Percentage of mosquitoes entering the hut that were found in the veranda. [b] Total number of mosquitoes collected in huts for each month indicated. [c] CI, confidence interval. [d] Two-proportion *Z*-test, $\alpha = 0.05$. [e] Deltamethrin and Imergard^{TM}WP mixed together and then sprayed.

3.3. Blood Feeding

A slight reduction in blood feeding was noticed during the 1st, 5th, and 6th months with ImG and during the 3rd, 5th, and 6th months with deltamethrin 62.5 SC-PE compared to the control (Table 3). No reduction in blood feeding was displayed by the mixture during the six-month trial (Table 3). However, it is important to point out that blood-feeding rates were in general very high across all treatments (>90%).

Table 3. Blood-feeding rates [a] of wild-type *An. gambiae* (s.l.) collected in the huts.

Treatment	Months	Total [b]	Proportion (%) [a]	95% CI [c]	*p*-Value [d]
Control	1	785	94.3	92.4–95.8	-
	2	331	95.5	92.6–97.6	-
	3	592	92.4	90.0–94.4	-
	4	796	95.5	93.8–96.8	-
	5	2084	97.4	96.6–98.0	-
	6	2355	96.1	95.3–96.9	-
Deltamethrin	1	716	94.8	92.9–96.3	0.713
	2	203	97.0	93.0–99.0	0.496
	3	542	95.9	93.9–97.4	0.016
	4	838	96.7	95.2–97.8	0.270
	5	1918	94.2	93.0–95.2	<0.0001
	6	2539	92.2	91.1–93.2	<0.0001
Imergard	1	645	89.3	86.6–91.6	0.0008
	2	204	98.0	94.9–99.6	0.186
	3	556	93.4	90.9–95.3	0.612
	4	719	95.3	93.4–96.7	0.956
	5	2098	95.5	94.5–96.3	0.0013
	6	2658	91.0	90.4–92.5	<0.0001
DeltaM+ ImG [e]	1	587	94.2	92.0–96.0	>0.05
	2	257	98.1	95.4–99. 5	0.137
	3	527	92.2	89.6–94.4	>0.05
	4	678	94.2	92.2–95.9	0.341
	5	1760	96.8	95.8–97.5	0.315
	6	2163	95.9	95.0–96.7	0.723

[a] Percentage of mosquitoes in the hut that were found in the blood-fed state. [b] Total number of mosquitoes collected in huts for each month indicated. [c] CI, confidence interval. [d] Two-proportion *Z*-test, $\alpha = 0.05$. [e] Deltamethrin and Imergard^{TM}WP mixed together and then sprayed.

3.4. Toxicity

Mortality rates after 72 h were low with deltamethrin 62.5 SC-PE ranging from 40% to 45% during the first four months and decreasing to about 25% for months five and six (Figure 3). On the other hand, high mortality rates were recorded over a period of six months for ImG ranging from 79% to 82%. The mixture of ImG and deltamethrin 62.5 SC-PE average mortality rate was 78%. The mortality rate for the treatments containing ImG alone, the mixture, and deltamethrin 62.5 SC-PE alone increased during the bioassay from 24 to 48 h and then from 48 to 72 h (Figure 3).

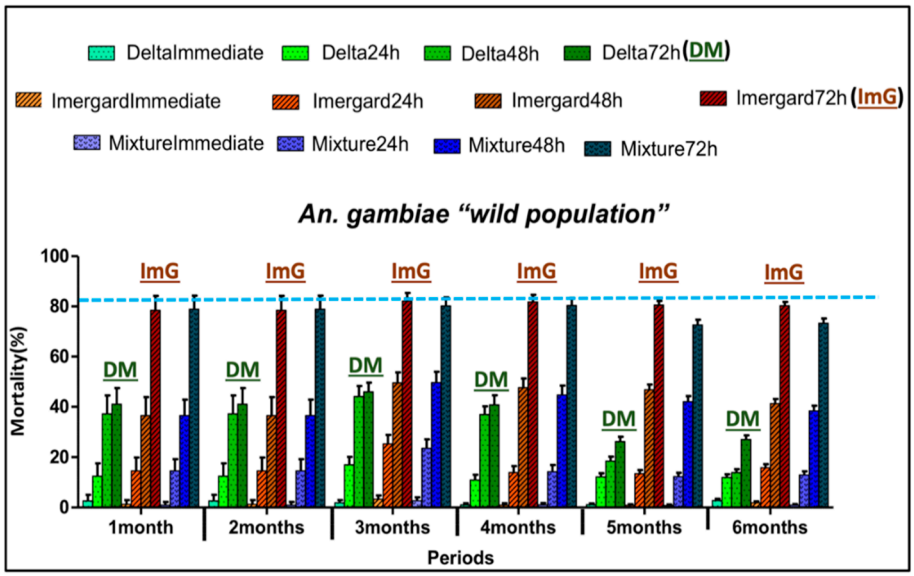


Figure 3. Monthly mortality rates of free-flying resistant *Anopheles gambiae* (s.l.) entering experimental huts in Cove, Benin. For each treatment, mortality was recorded at collection time (immediate) and then at 24, 48, and 72 h after collection. Error bars represent 95% confidence intervals. Delta and DM = Deltamethrin; ImG = Imergard^{TM}WP; Mixture = Deltamethrin + Imergard^{TM}WP. The blue dotted line indicates highest mortality rates obtained in mosquitoes collected in the huts.

3.5. Insecticide Residual Activity

The residual efficacy of each treatment was determined monthly by exposing pyrethroid susceptible and wild-type (pyrethroid-resistant) mosquitoes to treated walls. The WHO benchmark for bio-efficacy for a residual wall spray is 80% mortality [23]. For the susceptible "Kisumu" strain, at 48 h, the deltamethrin 62.5 SC-PE mortality was greater than 80% for the first four months and then decreased to about 45% in the last two months of the study (Figure 4). The huts treated with Imergard^{TM}WP showed mortality greater than 80% for five months with a slight decrease in the last month. Mortality remained above 80% for the mixture of ImG + deltamethrin 62.5 SC-PE for the entire study (Figure 4). With the wild *An. gambiae* (s.l.) population and for the same time point (48 h), the mortality was low and never exceeded 45% with deltamethrin 62.5 SC-PE (Figure 5). With ImG and the mixture, the mortality rates remained above 80% for five months and decreased to about 78% in the sixth month (Figure 5). Imergard^{TM}WP had 3.1 times greater residual activity than the deltamethrin 62.5 SC-PE alone for the first four months and had 5.7-fold greater efficacy at six months against wild-type mosquitoes. In these studies, mortality due to Imergard^{TM}WP alone or as a mixture with deltamethrin 62.5 SC-PE increased during the bioassay from 24 to 48 h. These results demonstrated that an MI made from volcanic rock is a suitable alternative to deltamethrin as a residual wall spray.

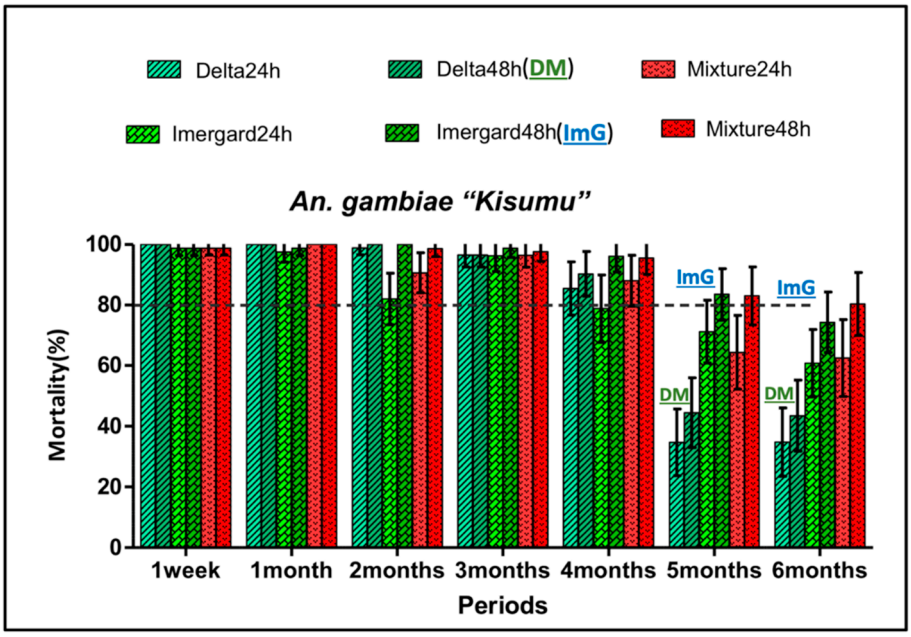


Figure 4. Residual mortality of Imergard^{TM}WP, deltamethrin, and their mixture following cone bioassays with laboratory susceptible *Anopheles gambiae* "Kisumu" strain in experimental huts in Cove, Benin. For each treatment, mortality was recorded at 24 and 48 h after a 30 min exposure to the walls. Error bars represent 95% confidence intervals. Delta and DM = Deltamethrin; ImG = Imergard^{TM}WP; Mixture = Deltamethrin + Imergard^{TM}WP. The gray dotted line indicates the 80% threshold requirement of the WHO for residual activity.

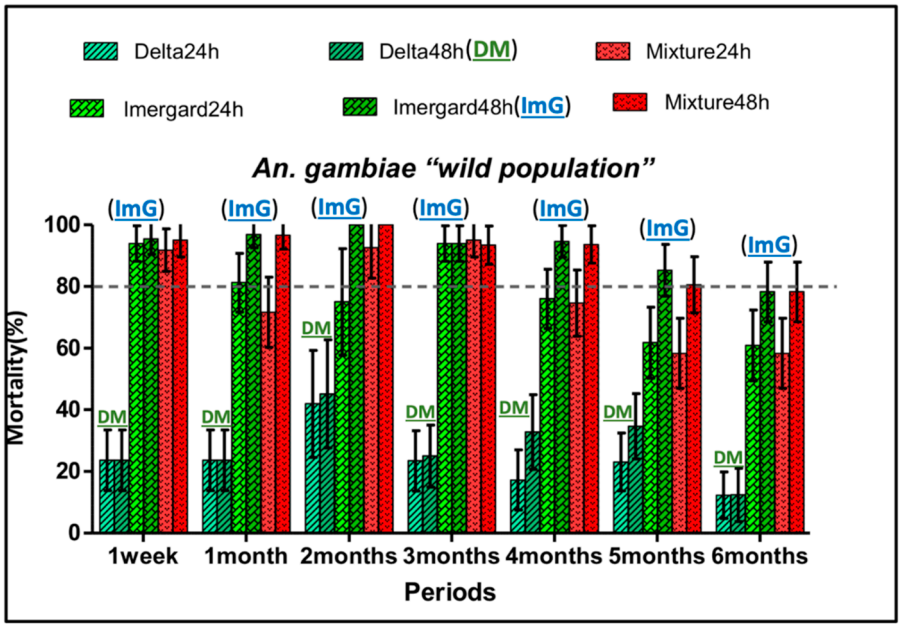


Figure 5. Residual mortality of Imergard^{TM}WP, deltamethrin, and their mixture following cone bioassays with wild resistant *Anopheles gambiae* (s.l.) in experimental huts in Cove, Benin. For each treatment, mortality was recorded at 24 and 48 h after a 30 min exposure to the walls. Error bars represent 95% confidence intervals. Delta and DM = Deltamethrin; ImG = Imergard^{TM}WP; Mixture = Deltamethrin + Imergard^{TM}WP. The gray dotted line indicates the 80% threshold requirement of the WHO for residual activity.

4. Discussion

Mosquito transmitted malaria parasites kill more people each year worldwide than any other single mortality factor, especially affecting children under the age of five and expecting mothers. The WHO African region accounted for 94% of all malaria deaths in 2018, while 67% (272,000) of all malaria deaths worldwide were children under five years [1]. Vector control with chemical-based, insecticide-treated bed nets and chemical-based insecticide (residual) wall sprays is critical to mosquito management and the reduction of malaria, the former including insecticide synergists. It is estimated that the use of insecticide-treated nets and the implementation of indoor residual spraying, taken together, averted over 517 million clinical cases of malaria from 2000 to 2015 [28]. Using a mathematical model to predict the health impact of various IRS products, Sherrard-Smith et al. [29] reported that Actellic® 300CS (an organophosphate) and SumiShield® 50WG (a neonicotinoid) grouped together averted up to 500 clinical cases per 1000 people per year. These predictions were determined for areas with moderate endemicity, high levels of pyrethroid resistance, low bed net use and 80% IRS coverage. In Benin, two rounds of bendiocarb (a carbamate) spraying in a community trial in 2008–2009 reduced biting rates of pyrethroid-resistant *An. gambiae* by over 80% and the parous rate by 70%, while none of the mosquitoes analyzed were infected (entomological inoculation rate = 0) [30].

Since the main pesticides currently used for bed nets are pyrethroid insecticides, the use of residual wall sprays with insecticides with a different mode of action like carbamates, organophosphates, and organochlorines are essential to managing mosquito insecticide resistance to bed nets. Despite this effort, insects are increasingly becoming more resistant to pyrethroids and demonstrating cross-resistance to other insecticides. In fact, insecticide resistance in malaria vectors has been documented for pyrethroids [6,8], organophosphates and carbamates [9,31], and organochlorines [32,33]. Currently, there is a significant effort to develop new chemical insecticides with new modes of action for bed nets and residual wall sprays. As of January 2020, 30 IRS products (24 pyrethroids, 1 neonicotinoid, 2 carbamates, 2 organophosphates, and 1 dual-active ingredient neonicotinoid-pyrethroid) as well as 19 ITN kits (18 pyrethroid-based and 1 dual-active ingredient alpha-cypermethrin-chlorfenapyr) have WHO prequalification listings (https://www.who.int/pq-vector-control/prequalified-lists/en, accessed on 24 April 2020). SumiShield® 50WG, for example, is a new IRS product (WHO prequalification achieved in October 2017) containing clothianidin, a neonicotinoid [22]. If alternatives are not found, we will lose mosquito control not only from wall sprays but also for bed nets, and the gains achieved in the last ten years in the reduction of malaria cases will be reversed.

The other consideration with insecticide-treated bed nets and residual wall sprays is the potential long-term use and exposure of people to chemical insecticides in the home. Contradictory results have been reported in the literature regarding the health effects of IRS insecticides, where some studies found adverse associations between exposure to insecticides such as DDT (dichlorodiphenyltrichloroethane, an organochlorine) and some pyrethroids and human health [34,35], whereas others found none [36,37]. However, the use of chemical insecticides is currently necessary because of the high risk of mortality from contracting malaria, and the insecticides being used have been proven safe. Not only are different modes of action needed for control but effective insecticides from natural sources with more specific action against the mosquito vector would be desirable.

In field trials in Benin, Africa, using WHO-approved huts (Figure 2) and standard WHO methods to evaluate residual wall sprays, ImG alone and as a mixture with deltamethrin (deltamethrin 62.5 SC-PE) was found to be significantly more effective than the pyrethroid alone and the untreated control. A deterrent effect (reduction in mosquito entry rates compared to the control) was found in the first four months (Table 1). The deterrent effect with ImG was not expected as the MI is odorless, and there was no obvious airborne MI in or exiting the experimental huts. One possible explanation is that some of the mosquitoes entering the hut made contact with a treated surface close to the hut entrance and became quiescent. When a mosquito moves, because of their proximity to the opening they have a better chance of exiting compared to the control huts. Because the entrance windows were engineered as one-way openings and deterrence levels were highly variable in different months, this explanation is most likely

not the mechanism for the calculated ImG deterrence found in some months. Entry reduction rates month to month were highly variable in our study, especially for ImG, and in most cases was less than 10% when compared to the control. It is most likely that entry reductions could simply be an artifact of the random geographical positioning of the control huts used to calculate deterrence compared to the ImG-treated huts and/or could be the result of environmental differences.

In two-choice repellency studies conducted in laboratory settings at North Carolina State University (NCSU), the malaria mosquito was not repelled by an ImG-treated surface [38]. Results here showed that once mosquitoes were in the hut, Imergard^{TM}WP did not repel the mosquitoes into the veranda (Table 2); there was no difference in exophily rates (the proportion of mosquitoes found in the hut veranda) between the control and the MI. This is consistent with the idea that an ImG surface is not repellent. The higher exophily rates observed with deltamethrin alone and the ImG–deltamethrin mixture is expected since pyrethroids are known to demonstrate mosquito spatial repellency [21,39].

No significant blood-feeding inhibition was observed for ImG, the pyrethroid, or the mixture of ImG with the pyrethroid (Table 3). In fact, blood-feeding rates were high in all treatments with >90% of the mosquitoes entering the huts acquiring a blood meal in both the treated and untreated huts. This is not uncommon for IRS treatments. No significant differences in blood-feeding rates were found between the treated and untreated huts during the evaluation of chlorfenapyr [21], clothianidin [20,22], and bendiocarb [40] in Benin.

Since there is no physical barrier, host-seeking mosquitoes often feed on the sleeper before landing on the walls to rest and digest their meal [20,22]. High percentages of blood-fed *An. gambiae* (s.l.) died after resting on the walls treated with Imergard^{TM}WP (79–82% average mortality) and the mixture (73–81%) during the 1–6-month study (Figure 3), thus confirming this technology has a significant potential as a new active ingredient for IRS. Furthermore, its residual mean activity as a stand-alone treatment was ≥80% mortality for at least five months for both susceptible (Figure 4) and resistant (Figure 5) mosquitoes; activity at six months was 76% and 78%, respectively (although the 95% upper confidence interval exceeds 80%). Although further studies are needed, it is possible that 80% mortality could be achieved past six months using the application rate in this study and/or increased even further by increasing the application rate. Examining application rates versus control duration for ≥80% mortality is needed in the future. The use of ImG at an optimum application rate for maximum control over time could lead to a reduction in the number of applications needed each year, a reduction in IRS application costs per home, and an increase in the number of homes that could be treated with the resources currently available. Furthermore, since ImG is not subject to thermal degradation, metal roofs can be treated, further improving efficacy. Based on the differences in mortality in the huts over six months, the MI killed 2.5-fold more mosquitoes (5517) than the pyrethroid (2180).

Imergard^{TM}WP showed a time-dependent increase in insecticidal activity against mosquitoes from 24 to 72 h. When a mosquito lands on a treated surface, a few particles of the product are statically transferred onto the insect's body. The insect's epicuticle contains lipids to prevent water loss [41,42]. Current understanding is that the lipid layer is disrupted by the mechanical insecticide. This increases water loss, disrupts the osmolality of the hemolymph, and results in death. The disruption of the lipid layer of the insect's cuticle is the presumed mechanism of action for MIs [16,17,41], and this process can take a few hours to a couple of days depending on the MI, the insect species, dose, environmental conditions (temperature and humidity), and the time since the last consumption of water or a blood meal. This delayed effect was also noticed with non-pyrethroid insecticides in IRS trials, e.g., with clothianidin (a neonicotinoid) [20] and chlorfenapyr (a pyrrole) [21]. It is therefore important that the WHO IRS insecticide evaluation guidelines, originally conceived for fast-acting insecticides, be reviewed or adjusted to account for the varying modes of action of new IRS alternatives currently being investigated.

Mosquitoes exposed to Imergard in the residual activity tests died at a faster and higher rate than the mosquitoes collected inside the huts. In the latter, mosquitoes entered freely and, once inside the huts, had the possibility of moving to different surfaces of the huts and into the exit traps. Some of

these surfaces were untreated. In the residual efficacy tests, mosquitoes were confined to a small space (WHO cones) presumably increasing their exposure to the treated surface for 30 min, and the mosquitoes may have acquired a higher amount of the insecticide in a shorter period. In laboratory studies, when *An. gambiae* were continuously exposed to Imergard in cones, 50% of the mosquitoes were dead in 5 h [38].

During the study, mosquitoes were not provided access to a 10% honey-in-water solution during the holding period. The sugar solution fed to mosquitoes used in the residual activity tests prior to their use in this study and the high blood-feeding rates (>90%) in mosquitoes collected inside the huts allowed them to survive without dying from starvation for the time course of the bioassays [43–45]. Blood feeding may have also decreased the rate of death during holding for the hut-collected mosquitoes. The MI could easily be washed away or become wet during prolonged interactions of the mosquito with a water source in their holding container. The close housing of mosquitoes within a few cm of a water source, comprised of a large wet surface area optimized for mosquito availability and feeding, creates artificial conditions not found under field conditions. For mechanical insecticides, water provisioning in confinement artificially blocks their mode of action. This is an important example where having rigid testing guidelines for evaluating new IRS technology requiring water for the mosquito might prevent the discovery and implementation of a new mode of action.

A legitimate concern could be that, in houses, access to wet environments might be possible and could reduce or prevent Imergard^{TM}WP action. However, this is not de facto true. After blood feeding, endophilic/anthropophilic mosquitoes seek a protected resting site inside the house (or sometimes outside) where they can digest the blood meal and develop eggs. At least 50% of *An. gambiae* females stay inside the home after a blood meal and invade interior walls, crevices, furniture, etc., for that purpose [46]. This stage of the gonotrophic cycle takes 1.8 days up to several days, where the duration varies depending on the mosquito species, the amount of blood consumed, and the environmental conditions [46–49]. Because blood digestion and egg maturation occur at rest with little to no interaction with a water source, it is likely that under natural conditions in a home the MI would have produced its lethal effect before the mosquito finished blood meal digestion and began to seek an oviposition site.

One concern about the use of a mechanical insecticide on the walls of a home is that human contact with treated surfaces might prematurely reduce the duration of control. A possible solution could be to treat only the upper part of the walls or target places where mosquitoes rest, such as crevices. Djenontin et al. [50] showed that covering only the upper one-third of the wall of huts with a carbamate-treated plastic sheeting (CTPS) provided equal or better efficacy compared to a traditional IRS application method using bendiocarb.

Improved toxicity and residual activity were obtained by mixing deltamethrin with ImG compared to deltamethrin alone especially for wild-type insecticide-resistant *An. gambiae* (Figures 3–5). Mixing two insecticides with different modes of actions to improve vector control even in areas of resistance was suggested before and recommended [6]; using two different modes of action could delay the evolution of resistance or reverse current levels of resistance. The WHO recommends that the two insecticides be co-formulated into a single product [6].

The poor performance observed with deltamethrin in our study (even though not surprising) is worrisome. Deltamethrin 62.5 SC-PE (a.i. 62.5 g/L deltamethrin; Bayer, WHOPES recommendations achieved in September 2013) is a polymer-enhanced suspension concentrate that came on the market only a few years ago and has been shown in several studies to be more effective than the standard deltamethrin WG25 against both susceptible and wild resistant mosquitoes [51,52]. The work here is suggesting that improving current active ingredients by formulation alone will not be sufficient in resolving resistance issues for the long term and that new active ingredients with a different mode of action are needed.

5. Conclusions

In summary, the use of a mechanical insecticide derived from volcanic glass represents a new paradigm for mosquito and malaria control. Applied as a stand-alone treatment or as a mixture with deltamethrin, Imergard^{TM}WP provided ≥80% control for five months for both susceptible and pyrethroid-resistant *Anopheles* mosquitoes in Africa with the likelihood of even longer-term activity. Activity at six months was 76% and 78%, respectively. Due to its new mode of action (in vector control), natural origin, safety (mammalian toxicity >10 g/kg) [18], abundance in nature, and low estimated cost, ImG should be an effective non-chemical alternative to current insecticides used in IRS. Imergard^{TM}WP, because of its mechanical mechanism of action, has a low probability of increasing insecticide resistance to chemical insecticides and therefore can be a powerful tool for integrated resistance management in Africa. The MI easily mixes with water and is applied using standard vector control spray equipment. This represents the first report on the practical use of a mineral in the field for mosquito control in Africa. More studies are needed to fully understand the potential of this new technology.

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insects

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Development of an Insecticide-Free Trapping Bednet to Control Mosquitoes and Manage Resistance in Malaria Vector Control: A New Way of Thinking

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Simple Summary: Insecticide resistance in mosquitoes reduces the effectiveness of malaria control interventions and has reversed the gains made in reducing malaria morbidity. Hence, new strategies are needed to mitigate the spread of resistance, preserve the efficacy of available insecticides, and restore the effectiveness of control. To combat resistance to insecticides in malaria mosquitoes, WHO recommends using long-lasting insecticide-impregnated mosquito bednets (LLINs) as well as the synergist piperonyl-butoxide (PBO). PBO enhances the insecticidal effect of the treated bednet. Unfortunately, decreases in performance of PBO-LLINs are now reported in some regions of Africa where mosquitoes are resistant to insecticides. Our objective was to develop an insecticide-free, mechanical solution that kills mosquitoes regardless of their insecticide resistance status, ultimately overcoming the problem of insecticide resistance. We designed and developed an insecticide-free mosquito trapping bednet for mass mosquito trapping and killing, the "T-Net", and we show its efficacy compared to a conventional LLIN in Africa. Mathematical models were also developed to predict T-Net efficacy in individual homes and at the community level.

Abstract: Mosquito-borne malaria kills 429,000 people each year with the problem being acute in sub-Saharan Africa. The successes gained with long-lasting pyrethroid-treated bednets are now in jeopardy because of wide-spread, pyrethroid resistance in mosquitoes. Using crowd modeling theory normalized for standard bednet architecture, we were able to design an attract–trap–kill technology for mosquitoes that does not require insecticides. Using three-dimensional polyester knitting and heat fixation, trap funnels were developed with high capture efficacy with no egression under worst-case laboratory conditions. Field testing in Africa in WHO huts with Gen1-3 T (trap)-Nets validated our model, and as predicted, Gen3 had the highest efficacy with a 4.3-fold greater trap–kill rate with no deterrence or repellency compared to Permanet 2.0, the most common bednet in Africa. A T-Net population model was developed based on field data to predict community-level mosquito control compared to a pyrethroid bednet. This model showed the Gen3 non-insecticidal T-Net under field conditions in Africa against pyrethroid resistant mosquitoes was 12.7-fold more efficacious than single chemical, pyrethroid-treated nets.

Keywords: malaria; vector control; insecticide resistance; long-lasting bednet; vector control failure; trapping bednet; permanet 2.0

1. Introduction

Malaria is the leading cause of morbidity and mortality in sub-Saharan Africa with 212 million cases annually estimated by the WHO and about 429,000 deaths each year [1]. Malaria prevention is mainly based on vector (mosquito) control, using insecticide indoor residual spraying (IRS) or long-lasting insecticide-impregnated mosquito bednets (LLINs) [2]. The efficacy of these measures depends primarily on the susceptibility of the mosquito to insecticides. An estimated 663 million cases of malaria have been averted in sub-Saharan Africa since 2001 as a result of the scale-up of malaria control interventions; 69% of these reduced cases were a direct result of the use of LLINs [3]. Unfortunately, mosquitoes have, in recent years, become resistant to insecticides including the pyrethroids, the only chemistry approved for bednets [4–6]. This resistance is threatening the efficacy of chemical-based, vector control [7–9]. There is an urgent need to develop new strategies to mitigate this problem.

Development of a new insecticide for bednets that is safe to human exposure every night for years, that must survive human handling each day and periodic washing, and that will not promote mosquito resistance and cross-resistance, is a challenge [10]. One solution is the reformulation of agricultural chemicals with completely different modes of action to that of the pyrethroids. This is a challenge since adult mosquitoes have been exposed to these insecticides applied to crop plants and as larvae developing in agricultural runoff; it has been hypothesized that mosquito resistance to bednets is a result of pesticide exposure from agricultural systems more than actual bednet use [11].

These challenges have led us to a different approach for mosquito control with bednets, a mechanical trapping and killing device. It is well known that mosquitoes are attracted to carbon dioxide gas and other human odors that are emitted from a warm body sleeping under the net, and these attractants rise through the top of the bednet [12]. Infrared video tracking has shown that about 75% of mosquitoes follow this odorant plume and are attracted to the top surface of the bednet [13,14]. We have taken advantage of this natural behavior to develop a mosquito trapping bednet, the T-Net, designed to trap and kill susceptible and resistant mosquitoes. The non-insecticidal T-Net has (i) a lower sleeping compartment and (ii) an upper mosquito trap compartment with funnels on the trap roof as mosquito entry points into the trap.

To optimize trapping performance, we hypothesized that mosquito movement is random outside of the trap compartment in the absence of attractants and visual cues. If we then consider mosquitoes as ideal gas particles, we can use the Maxwell–Boltzmann distribution [15] to develop a mathematical model to optimize the number, size, and positioning of funnels to maximize trapping efficacy that would also apply when someone is sleeping under the net. Different T-Nets derived from the model were assayed in walk-in simulated WHO huts using human subjects [16] and once proof of principle was demonstrated, further validated with wild-type, insecticide-resistant *Anopheles gambiae* mosquitoes under field conditions in Tiassale, Côte d'Ivoire, Africa. From the field data, we developed a second model to predict community-level mosquito control by the T-Net versus the most common bednet deployed in Africa.

2. Materials and Methods

2.1. T-Net Construction

The non-insecticidal T-Net has (i) a lower sleeping compartment and (ii) an upper mosquito trap compartment with funnels on the trap roof (with the small opening of the cone(s) pointing down into the trap compartment). The funnels serve as mosquito entry points into the trap. The sleeping

compartment of the T-Net was a standard (single-sleeper), non-insecticidal bednet (Huzhou Shuanglu Knitting Mill Co., Ltd., Jinhua, Zhejiang, China). The trap compartment was constructed from a second non-insecticidal bednet that was sewn on the original bednet roof (open end down) but where the height of the sleeping compartment (which is now the trap compartment) is reduced. Then knitted cones described later were sewn into holes made in the roof of the trap compartment. The detailed architecture of the T-Net and different iterations used in our research are discussed later. Once mosquitoes enter into the trap compartment through the funnels, they are blocked from reaching the sleeper by the roof of the first bednet.

2.2. Cone Knitting

T-Net prototyping was conducted using different knitted cones sewn into the roof of the trap compartment. Knitted cones can easily be constructed in any shape and size, are easily sewn into our T-Net, and can be folded into a flat, compact package for storing and shipping of bednets. Cones were knitted on a flat, computerized knitting machine, SWGN2 (Shima Seiki, Sakata Wakayama, Japan), where their dimensions for prototyping are easily changed by standard program options. Details on cone sizes are discussed later. The gauge of knitting machine was selected so that the resulting cone fabric would have openings between loops similar to those of traditional bednets. Like the construction of stockings or socks, cones were knitted in-the-round to make a 3D shape. Use of this construction method allowed us to alter the shape, depth, width, and height in all sections of the cone to meet our design demands. The knitted cones had three main parts—a large opening at the mouth (which was designed to allow for passage of mosquitoes into the cone), tapered cone walls (which were designed to direct the movement of mosquitoes into the trapping compartment), and a neck opening into the trapping compartment (the size of the neck was optimized to allow passage of mosquitoes and minimize egression from the trap). To prevent collapsing and flattening of the knitted cones, they were constructed with thermal bond fibers. This yarn is a polyester binder-spun yarn in which each fiber is composed of a sheath part (co-polyester) and a core (regular polyester). The bursting strength and durability of these cones described later were compared to conventional bednet material to make sure they would stand up to years of use.

2.3. Cone Bursting Strength and Durability Measurements

A burst test (James H. Heal TruBurst 2, ASTM D3786, James H. Heal & Co. Ltd., Halifax, UK) was used to measure the durability of the cones constructed versus bednet fabric; this is achieved by increasing the hydrostatic pressure across the textiles until they ruptured (Figure S1). The pressure was applied to a circular region of the fabric via an elastic diaphragm. The fabric sample was firmly held around the circular edge by pneumatic clamping. When the pressure was applied, the fabric deformed together with the diaphragm. The bursting strength corresponded to the maximum pressure supported by the fabric before failure.

The abrasion test (Figure S2) was used to assess the cone textile durability by friction compared to the bednet fabric. Abrasion resistance refers to the resistance of a fabric in the process of repeated friction with a defined, rough surface. To run the test, the fabric was loaded onto the lower plates of the abrasion tester (Maxi Martindale 1609, ASTM D4966, James H. Heal & Co. Ltd., Halifax, UK), and then abraded using oscillating circles. The assay end point was the duration and number of circles until the first hole appeared.

2.4. Tunnel Test for Assessment of Cone Efficiency

A modified WHO-tunnel test [17] was conducted to assess the rate at which mosquitoes would move through knitted cones and their potential use as part of a trap mechanism. The tunnel test was modified to include three compartments—a top compartment which operated as a mosquito release chamber, a middle compartment which operated as a trapping container, and a lower compartment that contained the arm of a human subject to attract host-seeking mosquitoes. The top

and middle compartments were separated by the knitted cone, and the middle and lower compartments were separated by a polyester net fabric to prevent the human subject from receiving mosquito bites (NCSU IRB approved protocol 16897).

The tunnel test was placed vertically to mimic the position of a cone on the top of the trap compartment, with the small opening of the cone pointing down. A human arm cleaned with distilled water and then air dried was inserted into the bottom compartment. Two replicates (the first one of 106 mosquitoes and the s using 50 depending on availability) of unfed five to eight-day-old, host seeking *An. gambiae* Kisumu-strain female adult mosquitoes were released into the top compartment. Mosquitoes used were reared in the Dearstyne Laboratory at North Carolina State University (Raleigh, NC) according to MR4 rearing protocols [18]. After 2 h from the time of release, the mosquitoes that remained in the top chamber were removed using a mouth aspirator and counted. The human arm was removed from the bottom compartment, and trapped mosquitoes were observed for an additional 2 h in the middle compartment. Mosquitoes moving from the middle to the top compartment was scored as egression.

2.5. Laboratory Walk-In Hut Trials

Laboratory walk-in hut trials were conducted in the Dearstyne Entomology Building. The rooms were 1.58 × 2.18 × 2.03 m (depth × width × height) and the tests conducted at 27 ± 1 °C and 65 ± 4% relative humidity. The T-Net was set up above a portable bed with the bottom of the sleeping compartment of the net on the floor of the room. Trapping was assessed with a human subject in the sleeping position under the net (NCSU IRB approved protocol 9067). Five- to eight-day old, unfed *An. gambiae* Kisumu-strain, adult female mosquitoes (described earlier) were used for these studies. For each test, the mosquitoes were released into the room but outside of the bednet. The number of mosquitoes released varied between 40 and 100 to determine if population density in the room had any effects on trap efficacy. The total number of replicates was six and these were conducted on different days. Trapping was conducted for 3 h during the photophase, since this mosquito strain has, for years, been fed with lights on. At the end of the experiment, mosquitoes were vacuum-collected from outside of the trap compartment (in the room) and counted immediately. To assess egression, trapped mosquitoes were not removed from the trap compartment. The number remaining in the trap were then counted after 30 min, 1 h, 2 h, 6 h, and overnight.

2.6. Field Trials in Africa

The study was conducted in experimental huts according to WHO protocols [17] in the municipality of Tiassalé (5°53′54″ N et 4°49′42″ W) in November 2018. The site is located in the south of Côte d'Ivoire, about 110 km north of the country's major city, Abidjan. The climate is tropical and characterized by four seasons—a long rainy season (March–July) during which two thirds of the annual rainfall occurs, a short dry season (July–August), a short rainy season (September–November), and a long dry season (December–March). The average annual rainfall is 1739 mm with an average annual temperature of 26.6 °C. The annual average relative humidity is around 70%. Rice production occurs in the lowlands of Tiassalé which facilitates the proliferation of mosquitoes throughout the year, and malaria is the leading cause of morbidity in the local population. The transmission of the disease is mainly due to *Anopheles coluzzii* (80%) and *An. gambiae* (20%) which have developed multiple resistances to insecticides [19,20].

The experimental field station of Tiassalé is made up of 18 standardized experimental huts (described in more detail later) situated close to the rice plantations. Each hut is 2.5 m long, 1.75 m wide, and 2 m high. The walls are made of concrete bricks and plastered with cement, while the floor is made of cement and the roof of corrugated iron sheets. A plastic cover is mounted underneath the roof as a ceiling to facilitate manual collection of mosquitoes. Each hut is built on a platform made of concrete surrounded by a water-filled moat that prevents entry of foraging ants. Entry of mosquitoes is facilitated through four window slits located on three sides of the hut. The slits are designed in such

a way as to prevent mosquitoes from escaping once they are inside the hut. Each hut is equipped with a veranda trap located on the fourth side, made of sheeting and screening to capture mosquitoes that would otherwise escape.

Four sleepers were trained and paid to sleep under the bednets. Each day, both sleepers and bednets were randomly positioned between four different huts. Trapping was conducted from 21:00 to 5:00, and the mosquitoes collected each morning after the sleep cycle was completed. Sleepers and bednets were rotated on a daily basis according to two Latin-square tables (Figure S3). The trial was conducted for 14 d. Each day, after mosquito collections, the huts were cleaned and the bednets rotated appropriately. Prior to the trial, sleepers were all vaccinated against yellow fever and were taken to the hospital to check for malaria parasites. All sleepers gave informed consent prior to enrolment in the study (CSRS IRB protocol #02-2011/MSLS/CNER-P). Within the hut, resting and dead non-trapped mosquitoes were collected from the room and in the veranda trap using 5 mL (12 mm × 75 mm) glass hemolysis tubes. Trapped mosquitoes were removed with mouth aspirators from the T-Net trap compartment by the field technicians. All mosquitoes were taken to the field laboratory and identified to genus level and scored as trapped-dead, trapped-alive, not trapped-dead, or not trapped-alive. Live mosquitoes were placed in cups and given access to a sugar solution for 24 h in the insectary at 25–27 °C and 70–80% relative humidity to assess delayed mortality.

Three different insecticide-free T-Nets (described in detail later) were evaluated in comparison to the WHO-recommended and widely-used Permanet 2.0-LLIN (PN2.0), a positive control. The PN2.0 was brand new and provided by the National Malaria Control program in Cote d'Ivoire.

The Kruskal–Wallis non-parametric test with the Conover–Iman multiple pairwise comparisons and the Bonferroni correction were used to analyze the data (alpha = 0.05). The analyses were conducted using the XLSTAT software package version 2019.4.1. [21] The overall blood-feeding rate was less than 3%.

3. Results

3.1. Crowd Model Applied to Mosquito Trapping

In our effort to develop a non-insecticidal bednet that kills mosquitoes by trapping, preliminary proof of concept studies was conducted to model mosquito interactions with a bednet designed for trapping. The model, if predictive of field conditions, could be used to evaluate different trap designs and make a better mosquito trap. The model could also provide a better understanding of how mosquitoes interact with bednets. If we treat a mosquito as an ideal gas particle, and the flight track is assumed to be random, the Maxwell–Boltzmann distribution [15] can be used for defining the random flight of mosquitoes at different velocities. This can further be applied to the dimensions of a standardized WHO approved, experimental research hut [16] as shown in Tiassale, Cote d'Ivoire (Africa) where our field work was conducted (Figure 1a,b). Figure 1c shows the process by which mosquitoes can fly from the interior of the hut through knitted cones into a trap compartment mounted above the sleeping compartment (Figure 1b). Mosquitoes that are in the trap compartment are blocked from the sleeper by the trap bottom, which is also the sleep compartment roof. Once the mosquitoes are trapped, they become exhausted and quiescent, and die from dehydration a few hours later.

Knitted cones were developed as access points to the trap compartment (Figures 1b and 2) with superior durability to standard bednet fabric (Figures S1 and S2). Use of knitting to make these cones allowed us to study different shapes, depths, widths, and heights to meet our design demands. The knitted cones had three main parts—a large opening at the mouth (which was designed to allow for passage of mosquitoes into the cone), tapered cone walls (which were designed to direct the movement of mosquitoes into the trapping compartment), and a neck opening into the trapping compartment (the size of the neck was optimized to allow passage of mosquitoes and minimize egression from the trap).

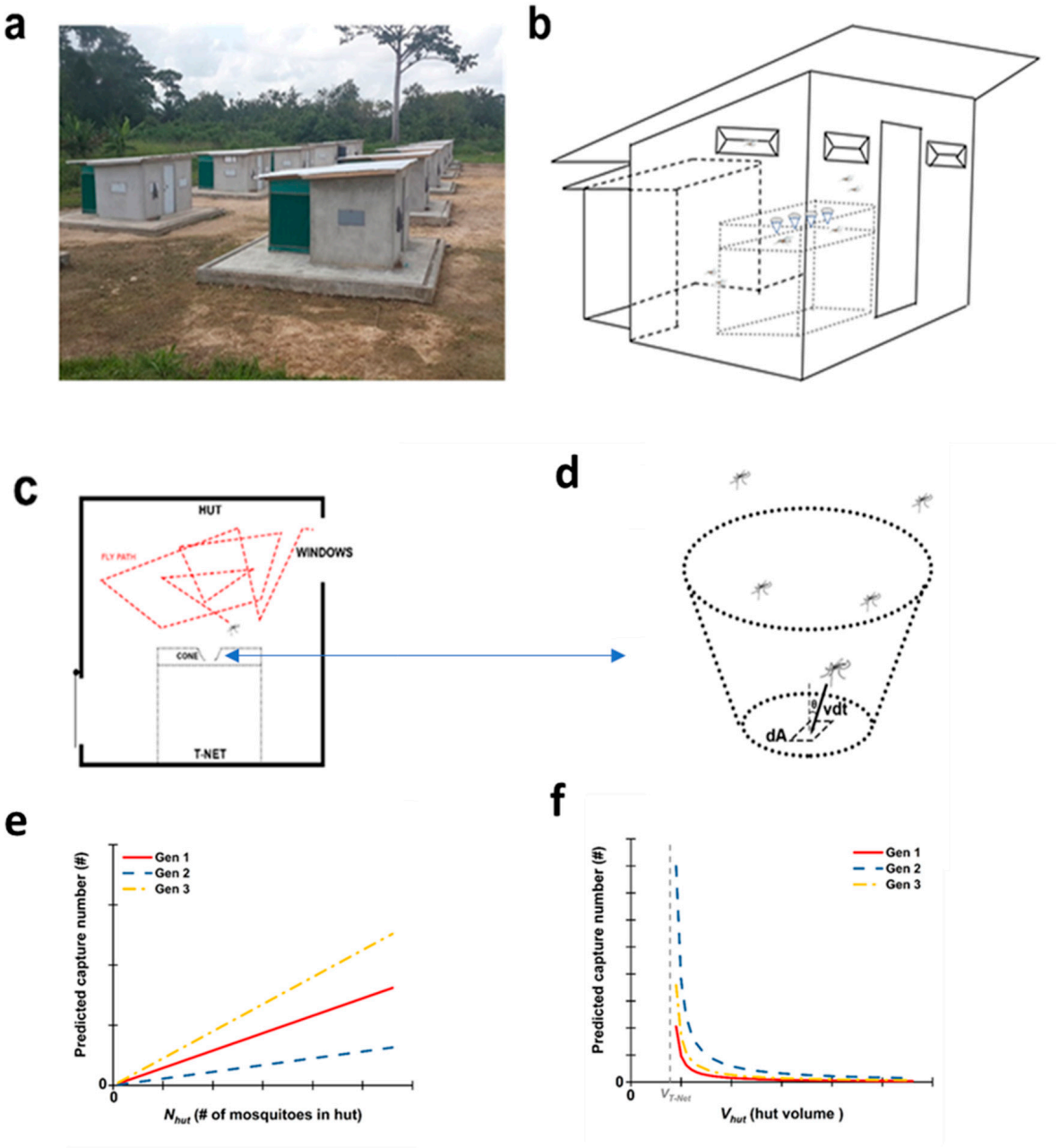


Figure 1. Principle and model of the T-Net. (**a**) Picture of experimental huts in the region of Tiassale in Cote d'Ivoire (Africa), where the field trials were conducted; (**b**) schematic of the hut and the T-Net deployed in the hut; (**c**) schematic of the mosquito flight track in the hut; (**d**) unit area on the bottom of the T-Net cone that captures the mosquito; (**e**) predicted capture number for Gen1, Gen2, and Gen3 T-Nets (see Figure 2a–c, respectively) with an uncertain number of mosquitoes flying into the hut; and (**f**) the predicted capture number for Gen1, Gen2, and Gen3 T-Nets when hut volume increases and the number of mosquitoes flying into the hut is constant.

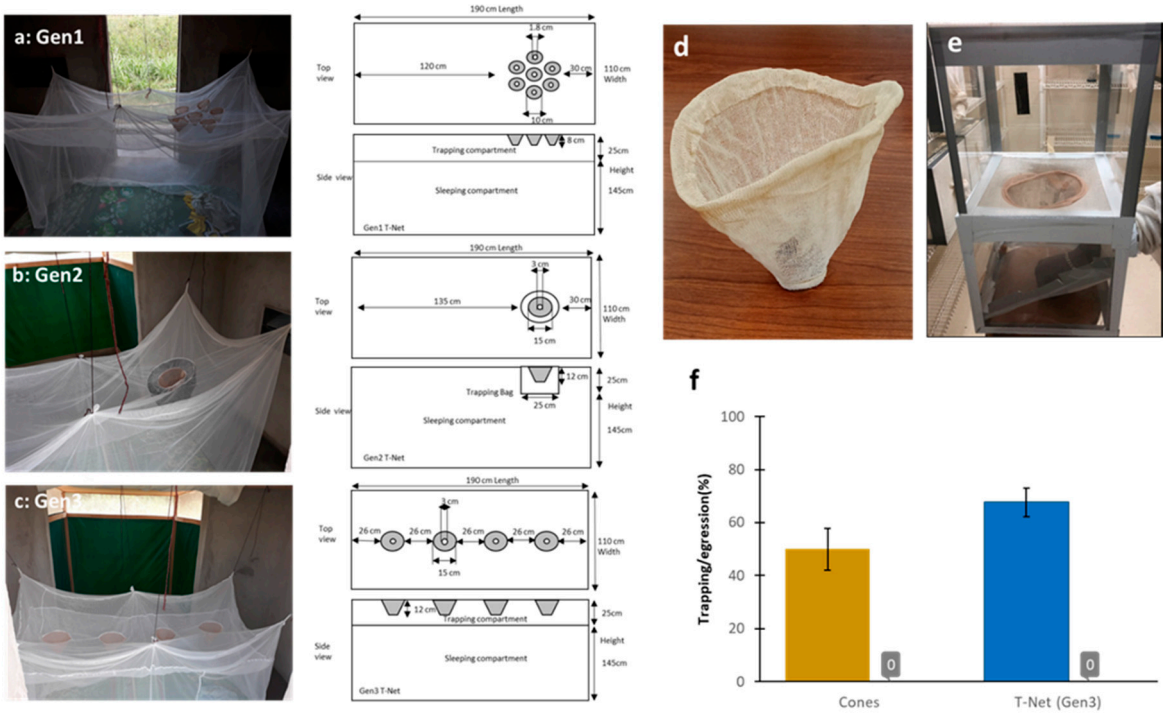


Figure 2. Different T-Net designs and testing. (**a**) Generation (Gen) 1 T-Net made of a circular aggregate of seven small cones, (**b**) Gen2 T-Net made with one large cone fitted on a trapping bag (**c**) Gen3 T-Net fitted with four large cones, (**d**) example of a knitted cone, (**e**) experimental tunnel apparatus to assess the trapping efficacy of a knitted cone, and (**f**) results obtained from the tunnel test for trapping efficiency of a large cone (on the left) and for the Gen3 T-Net in a laboratory hut experiment (on the right). The histograms represent the trapping rates, the bars on top are the 95% confidence intervals, and "0" are the exit rates (= egression) in the absence of host cues.

For model development, we assumed that (i) the flight track of mosquitoes is random (Figure 1c), (ii) the diameters of the cone into the trap compartment are large enough not to affect the flight path of other mosquitoes, and (iii) the trapping does not perturb the flight velocity of the remaining mosquitoes in the hut. Figure 1d assumes a hole area dA, a mosquito at a distance vdt from the hole, moving at a speed v and at an angle θ from the normal surface toward the area dA. All mosquitoes within a parallelepiped volume around the dA area moving toward the hole with speed v will pass through the top opening of the cone in the time interval dt. Therefore, the total number of mosquitoes flying through the dA area in the time interval dt is as follows:

$$N_A = \rho v cos\theta dA dt \quad (1)$$

where ρ is the mosquito density in the hut.

Assuming the distribution of individual mosquito velocities obeys the Maxwell–Boltzmann distribution, which was first defined and used for describing particle speeds in idealized gases, one integrated expression of the distribution relates particle density with average velocity. Similarly, the average trapped mosquito number per area per time is then:

$$\dot{N} = C\overline{v}\rho \quad (2)$$

where $\overline{v}$ is the average flight speed, ρ is the mosquito density in the hut, and C is a constant determined by the active behavior of the mosquito (1/4 in ideal gas theory). As mentioned above, most of mosquitoes fly to the top of the bednet and thus the work volume is as follows:

$$V_w = V - V_{net} - V_s \tag{3}$$

where V is the hut volume, V_{net} is the bednet volume, and V_s is the volume surrounding the bednet (except the volume on top of the bednet).

Assuming mosquitoes are distributed evenly on the top of the bednet, the trapped mosquito number for the T-Net in the testing time t is as follows:

$$N = C \times a \times \frac{N_{hut} \times \overline{v}}{(V - V_{net} - V_s)} \times t \tag{4}$$

where N_{hut} is the total mosquito number in the hut, C is a constant, and a is the bottom area of the cone. Thus, the T-Net model relates trapping number with container volume and flight velocity. If the number N_{hut} of mosquitoes in a hut is a variable and other parameters are constant, the predicted results of Equation (4) are shown in Figure 1e and if the volume V_{hut} of the hut is a variable and the number of mosquitoes a constant, the predicted trapping number for the T-Net is shown in Figure 1f for three different versions of the net that we investigated for model development and field validations (i.e., Gen1–3 prototypes shown in Figure 2a–c, respectively).

3.2. Construction of Different T-Nets and Model Prediction

The attraction of mosquitoes through the funnels into the trap compartment is hypothesized to be from insect attraction to carbon dioxide and other odors from human respiration. If this is the case, funnels should only be needed on the head end of the net. Based on this assumption, we designed and constructed the Generation (Gen) 1 T-Net with a circular aggregate of 7 cones (each cone having a 10 cm opening, an 8 cm depth, an inclination angle of 32 degrees, and a 1.8 cm small diameter); these cones were sewn around the midline of the long axis of the trap roof (a 25-cm-deep trap compartment), 30 cm from the sleeping end of the net (shown in Figure 2a). The model (4) predicted trapping rates shown by the regression curves in Figure 1e,f. To potentially increase the trapping rate and add additional functionality, a Gen2 T-Net was constructed (Figure 2b). In this case, one large cone 15 cm in the large diameter, 12 cm deep, and 3 cm in the small diameter, with a 51.3 degree of inclination angle, was fitted into a cylindrical trapping bag 25 cm deep and 25 cm in diameter fixed as before on the bednet roof 30 cm from the edge. The idea of using a trapping bag and a single cone was to reduce material costs for construction, make it possible to reduce the head space between the sleeper and the bednet roof (effectively increasing the odorant concentration on the bednet top) and to add practical functionality to the bednet design; the Gen2 net design could be used to retrofit any non-trap bednet including those already deployed in homes. From Equation (4), we predicted that this actually reduced the catch rate as compared to Gen1 (Figure 1e,f).

Intuitively, having the cones just above the head of the sleeper should produce the maximum trap rate if only carbon dioxide from the subject's breathing is at work. At the same time, reducing the overall cross-sectional area of the small openings of the cones and/or the number of cones might reduce the catch rate but also reduce the egression rate once mosquitoes were captured. There is also the possibility that odorants from the subject's body could be in play in trapping and that spreading cones in different areas on the net top might improve capture rates. To address the above in part, we increased the number of cones from one in Gen2 (Figure 2b) to four in Gen3 (Figure 2c). The Gen3 T-Net had four large cones (15 cm large diameter, 12 cm deep, 3 cm small diameter, and 51.3 degree of inclination angle; the same dimensions as Gen2), where the cones were aligned lengthwise along the center line of the bednet with the cones spaced 26 cm apart. All cones opened into a 25-cm-deep

trapping box (Figure 2c). Interesting, our model predicted an increase in the trapping rate over that for Gen1 and Gen2 based strictly on our model assumptions (Figure 1e,f).

Our model (4) predicted capture rates but not the possibility of egression once mosquitoes were trapped. Therefore, laboratory bioassays were conducted with lab-reared Kisumu strain, *An. gamibae*, adult females to examine egression rates across the large cones in the Gen3 net where the model predicted the highest capture rate. A high capture rate would not be optimal if the large cone and especially four large cones in Gen3 favored a higher egression rate. Studies were conducted using a modified WHO tunnel test [17] as shown in Figure 2e. The capture rate (with mosquitoes released into the top chamber and the subject's arm as shown in the bottom chamber) was 49.5% after 2 h. More importantly, the egression rate once the mosquitoes were captured was 0% 2 h after the arm was removed (Figure 2f). These results suggested that even though we added a greater overall area for mosquito entry into the trap in Gen3 which our model suggested would increase capture rate, the increased capture rate predicted did not also increase egression. The tunnel tests were also a worst-case scenario where the host cues were removed, and the trap volume was much smaller than that for the Gen3 trap compartment.

Before going to the field in Africa to validate our trapping net model, a laboratory test was conducted with the Gen3 T-Net in a walk- in incubator (same dimensions as field huts, see details in the Materials and Methods section) and with a human subject under the net in the sleep position. Susceptible, laboratory-reared *An. gambiae* Kisumu were released into the room after the subject entered the sleeping compartment of the T-Net. A total of 300 unfed adult, host-seeking female mosquitoes were tested over six replicates with an average of 50 mosquitoes per replicate (range = 41–85 females). The trapping rate in these studies ranged from 45.28% to 86.90% after 3 h with an average capture rate of 67.7% (± 20.5%, 95% CI), (Figure 2f). No mosquito egression from the trapping compartment was observed at 30 min, 1 h, 2 h, 6 h, or overnight) for any of the replicates (Figure 2f). These results demonstrated proof of concept that the predicted best-performing Gen3 T-Net, under laboratory conditions simulating WHO experimental hut conditions at our field site in Africa, was efficacious in mosquito capture and retention.

3.3. Field Performance of Gen1, Gen2, and Gen3 T-Nets Against Insecticide Resistant, Wild, Free-Flying Mosquitoes

To fully validate our model (4) for predicting the trapping efficacy for the T-Net, field trials were conducted comparing Gen1, Gen2, and Gen3 bednets that were compared to a positive control, the Permanent 2.0 (PN2.0)-LLIN (the most used LLIN in Africa). The other objective of the field trials was to evaluate the efficacy of the T-Nets compared to a popular LLIN (Permanet 2.0) and determine if the technology is reasonably practical as a mosquito control strategy or whether more research is needed to further optimize the T-Net. These studies were conducted in WHO-approved experimental huts [16] in the locality of Tiassale where wild *An. gambiae* malaria vectors are resistant to insecticides [19,20], with human subjects sleeping under the nets each night. Three entomological parameters [22,23] were measured: (i) the mean entry rate of mosquitoes per hut per night, (ii) the mean exit rate per hut each night, and (iii) the mean mortality rate per hut per night. For the T-Nets, we scored the trapped mosquitoes as alive or dead each morning but in practice, trapped mosquitoes are ecologically dead, i.e., they are quiescent, no longer able to blood-feed and typically die before the next sleep cycle. Only the *An. gambiae* s.l. were considered in this study. *Culex* spp. were also collected but in a low number (less than 100 total), too low for a valid separate analysis from that of *Anopheles*.

Mean entry number: A total of 53, 139, 175, and 156 *An. gambiae* s.l. mosquitoes were collected over 14 nights (representing 14 observations = 14 replicates), respectively, for PN2.0, Gen1, Gen2, and Gen3 nets (Table 1). The mean number of mosquitoes entering each hut per night was 3.8 (σ= 2.96) for PN 2.0 (the lowest entry rate in the field trial), followed by 9.9 (σ = 10.1) for Gen1, 12.5 (σ = 10.5) for Gen2, and 11.1 (σ = 8.3) for Gen3. The differences were not statistically significant between Gen1,

Gen2, and Gen3, but a significant difference was observed between PN 2.0 and each of the T-Nets ($p < 0.05$) (Figure 3a).

Table 1. Results from field evaluations in Tiassale (Cote d'Ivoire) where mosquitoes are resistant to insecticides of Gen1, Gen2, and Gen3 T-Nets compared to the Permanet 2.0 (PN2.0)-LLIN. Shown are the total mosquitoes caught, mean entry rate, mean exit rate, and mean mortality rate per hut per night for *An. gambiae* s.l mosquitoes after 14 observations.

			Entry #		Exit Rate (%)		Killing Rate (%)	
Bednet	**# obs.**	**Total Entry (#)**	**Mean ***	**Std dev.**	**Mean ***	**Std dev.**	**Mean ***	**Std dev.**
PN2.0	14	53	3.8a	3.0	77.2a	29.4	14.4a	18.0
Gen1	14	139	9.9b	10.2	47.3b	27.8	39.1b	30.6
Gen2	14	175	12.5b	10.6	45.7b	30.6	39.9b	26.1
Gen3	14	156	11.1b	8.3	26.8b	20.7	61.9c	22.1

obs., number of observations; * values of the same column not sharing the same letters are statistically significant ($p < 0.05$); Std dev., standard deviation.

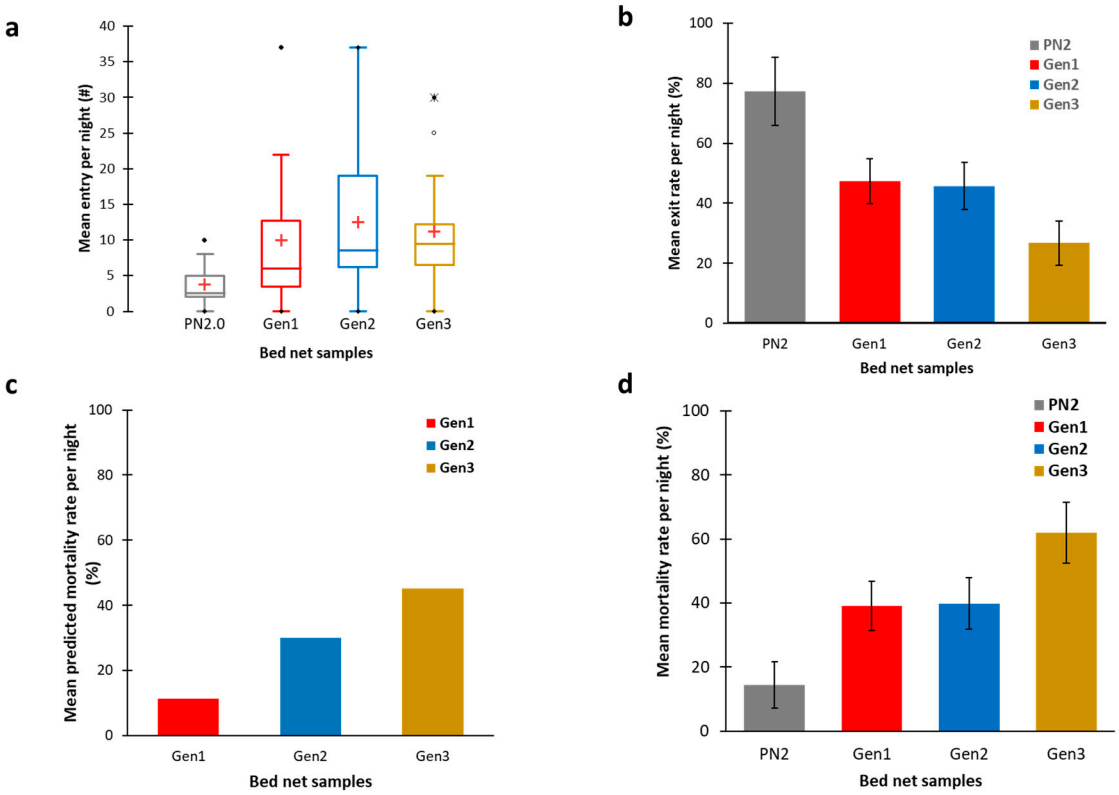


Figure 3. Performance of Gen1, Gen2, and Gen3 insecticide-free T-Nets in comparison to the Permanet 2.0 LLIN (PN2.0) in Tiassale (Cote d'Ivoire) where mosquitoes are resistant to insecticides. The nets were randomly allocated to four huts. Nets and sleepers were rotated each night. Up to 14 observations were made. (**a**) The mean number of mosquitoes entering each hut per night, (**b**) the mean exit rate per hut per night, (**c**) the mean predicted killing rate of the T-Net, and (**d**) the mean mortality rate per hut per night (trapped = dead and ecologically-dead mosquitoes). The bars on top of the histograms represent the 95% confidence intervals.

Mean exit rate: As expected, the PN2.0 not only showed the lowest entry rate but also the highest mean exit rate of mosquitoes per night, 77.2% ($\sigma = 29.4$) and was statistically significantly different from that of Gen1, Gen2, and Gen3. In Gen1 and Gen2, we recorded a mean mosquito exit rate per night of 47.3% ($\sigma = 27.7$) and 45.7% ($\sigma = 30.6$), respectively, and in Gen3, the exit rate was 26.7% ($\sigma = 20.7$)

which was the lowest exit rate. No statistically-significant differences in exit rates were observed between the three T-Nets (Table 1, Figure 3b).

Mean mortality rate: The lowest mortality rate (Figure 3c) occurred with PN2.0, 14.3% (σ= 17.9). It was statistically significantly lower than for all of the T-nets. The mortality rates for Gen1 and Gen2 were 39.1% (σ = 30.5) and 39.8% (σ = 26.0), respectively, with no statistically-significant differences between these two nets ($p > 0.05$). The highest mean mosquito mortality rate per hut per night was for Gen3 at 61.9.5% (σ = 22.1). This difference was higher than that of Gen1, Gen2, and PN.20 ($p < 0.05$).

3.4. T-Net Model Validation

Base on the tunnel test, *C* in Equation (4) was calculated by the condition parameters where the tunnel test cage dimensions were 30 cm in length, 30 cm in width, and 30 cm in height. The cone trap diameter was 3 cm. Thus, *C* was 0.00783. With these data, we predicted the trap number for Gen1, Gen2, and Gen3, respectively, at 2.87, 1.41, and 5.02 corresponding to trapping rates of 28.9%, 11.3%, and 45.06% (Figure 3c). The relative trap efficiency between Gen1, Gen2, and Gen3 calculated from the model was in agreement but underestimated the actual trap efficacy in the field trials (Figure 3d). This was expected since the model assumptions did not take into consideration the attractants from the sleeper. For the Gen2, there was a much greater trap rate than predicted, suggesting that cone positioning above the sleeper head was likely responsible for a greater number of trapped mosquitoes than the other cones in Gen3. At the same time, the higher efficacy of Gen3 where cones run along the long top axis of the net, suggests that odorants from other parts of the body are important in trap efficacy.

3.5. Model for Community-Level Mosquito Control Using the T-Net

Gen1 and Gen2 T-Nets had a 2.7-fold greater kill rate than PN2.0 while Gen3 had a 4.3-fold greater kill rate than the permethrin-treated (PN2.0) positive control. However, there is more to consider in comparing these different bednet technologies, and the T-Net impact at the community level is actually much greater than these data suggest. The deterrence rate (diminution in the rate of entry due to the chemical) was much lower and the repellency rate much higher for PN2.0 compared to the T-Nets (Table 1, Figure 3). If the mosquitoes do not enter the hut because of deterrence and if they enter the hut but are repelled before receiving a lethal insecticide dose from the bednet, these insects are not controlled. The model (Equation (5)) described in Figure 4 takes these factors into consideration to calculate "overall relative mosquito control":

$$\text{Overall relative mosquito control } = \frac{M_u}{(1-D) \times M_i} \quad (5)$$

where M_u is the mortality caused by the untreated T-Net (%), M_i is the mortality caused by the insecticide treated net PN2.0 LLIN (%), D is the deterrence rate $\left(D = \frac{(E_u - E_i)}{E_u} \times 100\%\right)$, E_u is the mean entry number for the untreated T-Net, and E_i is the mean entry number for the insecticide-treated PN2.0 LLIN.

Using this model (5), Gen3 was 12.6-fold more efficacious than PN2.0, and Gen2 and Gen1 were 9.1-fold and 7.1-fold, more efficacious, respectively (Figure 4).

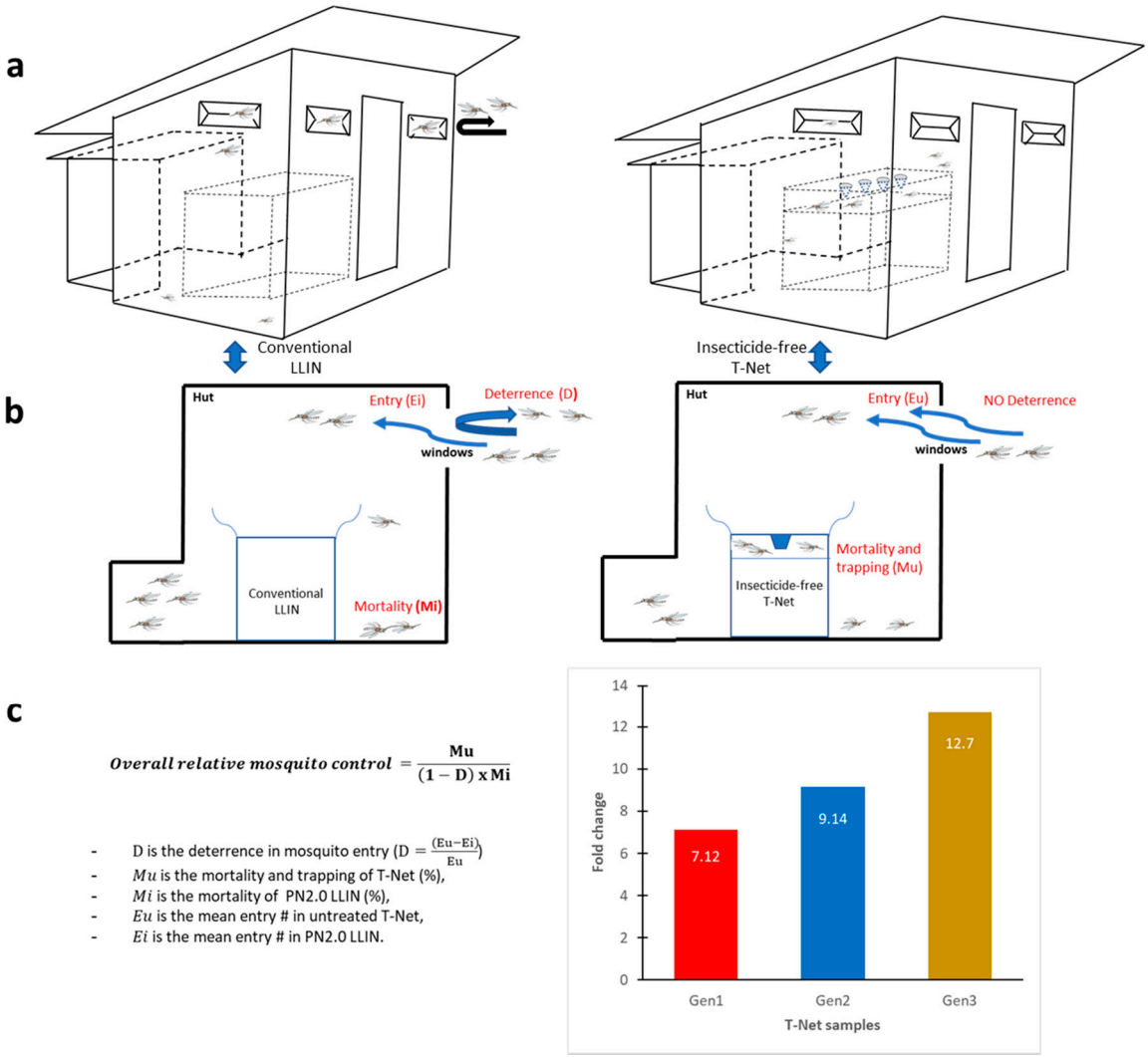


Figure 4. Overall relative mosquito control. (**a**) Schematic illustration of the experimental hut with either a LLIN or a free-insecticide T-net, (**b**) the different parameters used to define the model, and (**c**) overall relative mosquito control. The histograms show the increased mosquito mortality for Gen1, Gen2, and Gen3 T-Nets compared to the PN2.0-LLIN.

4. Discussion

Malaria is a devastating disease, and vector control is a crucial factor in saving lives. However, the situation has become critical because mosquitoes have become resistant to core intervention tools such as LLINs and IRS. Tackling insecticide resistance by developing new approaches for vector control is imperative to controlling malaria, and we need to think "out of the box".

The current model-driven insecticide-free trapping bednet is a paradigm shift in malaria vector control and could be a simple solution to the problem. Its functionality is based on the attraction and trapping of mosquitoes regardless of their insecticide-resistance status. This proof-of-concept study describes, for the first time, the efficacy of a trapping bednet as a malaria vector control strategy, that addresses the problem of insecticide resistance without the need to develop new chemistry. The advantage of the model developed is that we can predict the mosquito trapping rate before going into the field for testing; this facilitates additional innovation in the future.

As commonly found in experimental hut trials in which non-insecticide and insecticide-treated products are compared [22,23], we found higher mosquito entry rates in huts with T-Nets (free of insecticides) than that observed with the PN2.0-LLIN. Similarly, the exit rate was higher

for the PN2.0-LLIN than that of the T-Nets. This is likely due to the excito-repellency property of the insecticide in the nets. The excito-repellency effect can be perceived as beneficial to the extent that it keeps some mosquitoes away from the sleeper, and thus provides personal protection to the net user. However, at the community level, the benefit of the excito-repellency effect can be nullified or even contribute to behavioral resistance and increase outdoor malaria transmission as mosquitoes are repelled outdoors and are not killed [24,25]. Direct observation of our field results showed a 2.7 to 4.3-fold increase mortality for the T-Nets compared to the PN 2.0-LLIN. However, due to mosquito deterrence and repellency for the insecticide-treated bednet, community protection was estimated by our new model to be 13-fold greater for the Gen3 T-Net compared to PN2.0. Thus, mass mosquito trapping from the long-term use of T-Nets could lead to a decline in the vector population where transmission is no longer stable. This population decrease is even more likely in the context of insecticide resistance where mosquito mortality is lower than expected by LLINs.

The T-Net exploits the natural behavior of mosquitoes, which principally interact with the roof of the mosquito net [13,14]. Therefore, the positioning of the traps on the top of the net promotes entrapment of mosquitoes. This is especially true for insecticide-free T-Nets which do not cause repellent effects. Untreated T-Nets can therefore be an asset for vector control as they trap and kill mosquitoes irrespective of their insecticide-resistance status. Nevertheless, the addition of a trap compartment to an existing LLIN can also enhance the killing performance of the net (data now shown). An insecticide-free bednet for malaria vector control is far from being adopted because of regulatory requirements that focus on the use of insecticides in bednets and the possible requirement for epidemiological data to prove the public health value. Although the idea of an insecticide-free bednet for malaria vector control was recently mentioned [26], an interim solution could be a hybrid version of the T-Net consisting of an insecticide-free trap compartment mounted on currently-used LLINs. In this case, community protection would be provided both by the insecticidal effect and the mass trapping of mosquitoes.

Studies have shown that the escalation in resistance to insecticides closely matches the introduction of insecticide-treated nets. To combat resistance to insecticides, the trend today is to use LLINs combined with the synergist piperonyl-butoxide (PBO) [27]. The PBO inhibits enzymatic activity of mosquito insecticide detoxification enzymes, and therefore enhances the insecticidal effect of the treated bednet. Unfortunately, decreases in performance of these mosquito nets are starting to be observed in some regions of Africa where mosquitoes are resistant [22,27]. Because the T-Net is insecticide-free, it does not exert any insecticide-resistance selection pressure on mosquitoes and is not impacted by insecticide resistance. The exclusive and prolonged use of the T-Net in areas that are endemic for insecticide resistance should eventually lead to a decrease in the level of resistance and thus facilitate the reintroduction of insecticides.

Though we have not analyzed the escape rate for trapped mosquitoes in the field, our lab assays suggest the egression rate will be low or zero. We also have found, in laboratory conditions, that trapping elicits a mosquito escape response that rapidly leads to exhaustion and death from dehydration and starvation. The dead mosquitoes in the trap compartment are not easily sighted by someone sleeping under the net, and trapped dead mosquitoes are easily removed by traditional washing methods in Africa. This finding suggests that removing mosquitoes from the T-Net trap compartment is not an issue. They could also be removed by net shaking when the funnels are pulled out. However, we believe that the view of mosquitoes in the trap compartment can be an asset to the extent that it could encourage the use of bednets.

Sampling of mosquito populations for transmission studies or evaluation of other vector-control tools such as IRS could also be conducted with insecticide-free T-Nets. Various anthropophagous mosquito sampling tools exist but are complex to build [28–30]. If compared to these methods, the simple construction mode of the T-Net, which uses inexpensive materials, makes it an easy-to-assemble and a less expensive sampling tool. It would be interesting to consider a comparative study of its effectiveness as a sampling method vis-à-vis other methods.

We have not conducted in depth manufacturing cost studies, yet improvements for mass production at low cost are underway. For example, the cones on the roof of the T-Net can simply be mass produced by heat stamping. We anticipate a satisfactory cost range of USD 2–4 [31] per T-Net, which is the current range for LLINs on the market. Furthermore, because there is no new chemistry to develop, the route to market should be rapid compared to an insecticide-treated bednet where new chemistry must be proven safe. It would also be useful to survey public opinion about the use of an insecticide-free bednet versus an LLIN where efficacies for both in a worst-case scenario are equal.

5. Conclusions

Proof of concept has demonstrated that a non-insecticidal bednet that kills mosquitoes by mechanical methods, in this case trapping, can be an efficacious "new way of thinking" vastly different from the accepted standard of using bednets treated with insecticides to prevent mosquito biting and to kill mosquitoes in Africa and other places. In this study, the trapping (T)-Net demonstrated a 4.3 fold greater kill rate in experimental huts and a predicted 12.7 fold greater control rate at the community level for insecticide-resistant *Anopheles* mosquitoes than the insecticide treated bednet used as a control. The non-insecticidal T-Net should be considered as another possible tool for malaria control as a stand-alone system, and when constructed from insecticide treated textiles (an insecticide treated T-Net) could be used to improve the efficacy of insecticide treated bednets in general; in both cases, these applications should help reduce the evolution of mosquito resistance to both old and future new insecticide chemistries in bednets.

6. Patents

A patent has been awarded in Africa for the T-Net (Patent No. 17063 in BOPI #05BR/2015). Additional IP for the T-Net has been filed by North Carolina State University and is currently pending at the Patent Cooperation Treaty (PCT) in Geneva.

Supplementary Materials: The following are available online at http://www.mdpi.com/2075-4450/11/11/732/s1, Figure S1: Knitted cone fabric and conventional polyester 100-dernier bednet fabric were subjected to an increasing hydrostatic pressure to measure bursting strength. The pressure was applied to a circular region of the fabric sample via an elastic diaphragm. The bursting strength corresponded to the maximum pressure supported by the fabric before failure. (a, left) is the burst strength, (b, middle) is the displacement, and (c, right) is the burst time. The burst strength, displacement, and burst time were higher for the cone fabric than for the bednet textile. Figure S2: Results from the abrasion test on the knitted cone fabric compared to conventional polyester 100-dernier bednet fabric. The assay end point was the duration and number of circles until the first appearance of a hole. The run speed used was 1000 circles (cycles) per 20 min. The abrasion duration of the cone fabric was 8.67 h, corresponding to more than 25,000 circles, whereas for the bednet sample, the duration was 2.67 h and less than 10,000 circles. Thus, the cone fabric presented a better abrasion resistance than the bednet fabric. Figure S3. Ballot box draws were used to assign bednets and sleepers to each hut. Two different Latin-square table designs were used. The first (top) was for bednet rotations, and the second (bottom) was for sleepers. Bednets and sleepers were then rotated according to the above order generated by a Latin-square table generator. Every 4 days, new ballot box draws were conducted to redistribute bednets and sleepers to huts.

Author Contributions: C.S.M. initiated the T-Net concept, contributed to the design of Gen-1, Gen-2, and Gen-3 T-Nets, carried out laboratory studies, supervised experimental field trials, analyzed the data, and drafted the manuscript. K.L. proposed and verified the T-Net model. A.J.W. designed and conceived the knitted cones. B.K.F. helped supervise the field study. M.G.M., R.M.R., and C.S.A. worked to design the T-Nets, approved the study design, and edited and improved the manuscript. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare a conflict of interest. C.S.M., A.J.W., M.G.M., R.M.R., and C.S.A. are both authors and inventors. A patent on the T-Net is currently pending at the Patent Cooperation Treaty (PCT) in Geneva. The T-Net was licensed to the company, Vector Textiles for taking the technology to market.

Ethical Approval and Consent to Participate: The ethics committee which approved the research study in experimental huts in Cote d'Ivoire is the Comite National d'Ethique et de la Recherche of the Ministry of Health

of Cote d'Ivoire under protocol #02-2011/MSLS/CNER-P. All the sleepers provided written informed consent to participate. Laboratory experiments at the North Carolina State University (NCSU) were done in compliance with IRBs approved by NCSU under NCSU IRB protocol #16897 and NCSU IRB protocol #9067.

Availability of Data: All data generated or analyzed during this study are included in this published article.

List of Abbreviations: T-Net, trapping bednet; min, minute; WHO, World Health Organization; LLIN, long-lasting insecticidal net; PN2.0, Permanet 2.0; Gen, generation; NCSU, North Carolina State University; Std dev., standard deviation.

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New thinking for filth fly control: residual, non-chemical wall spray from volcanic glass

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Abstract. Filth flies are of medical and veterinary importance because of the transfer of disease organisms to animals and humans. The traditional control methods include the use of chemical insecticides. A novel mechanical insecticide made from volcanic glass and originally developed to control mosquitoes (Imergard™ WP; ImG) was investigated for control of adult grey flesh flies, *Sarcophaga bullata* (Parker), secondary screwworms, *Cochliomyia macellaria* (F.), and house flies, *Musca domestica* L. In a modified WHO cone test device, the time to 50% mortality (LT_{50}) when applied at 5 g/m^2 (tested at 30 °C and 50% relative humidity (rH)) was 7.1, 4.3 and 3.2 h, respectively. When knockdown was included, the LT_{50}s were 5.5, 1.5 and 2.8 h, respectively. Application rates of 1.25 and greater g/m^2 had the shortest LT_{50}s. The time to the LT_{50} increased for *M. domestica* as rH increased, but ImG was still active at the highest rH tested of 70%. Scanning electron micrographs showed ImG was present on all body parts, unlike that for mosquitoes where it was found mostly on the lower legs. These first studies on the use of Imergard WP against flies suggest this could be an alternative method for filth fly control.

Key words. Grey flesh fly, house fly, Imergard™ WP, mechanical insecticide, secondary screwworm.

Introduction

Filth flies in the families Sarcophagidae, Calliphoridae and Muscidae breed in unsanitary organic materials, e.g., human food waste, vegetable waste, faeces and carrion (Mingchay *et al.*, 2014; Onwugamba *et al.*, 2018). These insects are of medical and veterinary importance as mechanical carriers of pathogens that cause human and animal diseases and also are a nuisance (Sukontason *et al.*, 2006; Deguenon *et al.*, 2019a, 2019b). Flies transfer pathogens by their contaminated appendages, defecation and regurgitation including the transfer directly to food, urban structures and even to crop plants (Wasala *et al.*, 2013). Grey flesh flies, *Sarcophaga bullata* (Parker), and secondary screwworms, *Cochliomyia macellaria* (F.), cause human and animal myiasis (James, 1947; Hall, 1991). House flies, *Musca domestica* L., are associated with over 100 pathogens (Khamesipour *et al.*, 2018). The food contamination by *M. domestica* with enteric bacteria, e.g., *Escherichia coli* (Szalanski *et al.*, 2004) and *Salmonella spp*. (Pava-Ripoll *et al.*, 2012), is a public health problem.

The traditional control of filth flies greatly depends on the use of chemical insecticides including pyrethroids, organophosphates, carbamates and others (Khan *et al.*, 2013). However, the development of resistance has been a severe problem (Scott *et al.*, 2013). The broad and incorrect use of chemical insecticides also increases the impact on non-target organisms and the

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environment. Hence, it is crucial to search for environmentally friendly and effective substitutes.

Mechanical insecticides (MIs) are industrial minerals that provide a physical mode of action to control arthropod pests. MIs have been shown effective against numerous pests, i.e., beetles (Showler, 2002), whiteflies (Liang & Liu, 2002), bed bugs (Doggett *et al.*, 2008) and cockroaches (Faulde *et al.*, 2006). The potential health risks to MIs have been considered before (McLaughlin *et al.*, 1997; Rice *et al.*, 2001).

The authors recently discovered a new mineral-based mosquitocide derived from 100% expanded perlite, now being commercialized as Imergard™ WP (ImG) by the company, Imerys (Deguenon *et al.*, 2020a, 2020b). ImG tested in Africa as an indoor residual wall spray for mosquito control was effective in Phase II trials for 6 months (reaching the 80% control threshold) while the pyrethroid alternative failed all 6 months. Perlite is an alumina-silica, volcanic rock typically formed by the hydration of obsidian. The expanded perlite is prepared by heating the volcanic rock to 760–980 °C; this increases its volume 4–20-fold (Maxim *et al.*, 2014). Expanded perlite is used in the construction industry as insulation material (Stanislavovich *et al.*, 2013) and in horticulture as a soilless, plant growth medium (Silber *et al.*, 2010). Perlite is also classified as 'Generally Recognized as Safe Substances' by the US Food and Drug Administration (FDA) for uses like filter aids in juice processing (Wang *et al.*, 2017) and a whitening agent in toothpaste (Joiner, 2006) with a Food Chemical Codex as a filter aid in the production of beverages around the world (FCC, 2020). Perlite has never been considered for use in the control of filth flies. MIs in general are chemically inert materials of interest for many years for insect control; their widespread use so far has been limited. The mode of action of MIs is thought to be a physical action that damages the insect's cuticle by abrasion and/or lipid absorption, resulting in disruption of water balance (Korunić *et al.*, 2016). This paper is a proof-of-concept study to determine if ImG developed for mosquito control could be used to control other medically important insects like filth flies.

Materials and methods

Insects and mechanical insecticide

Sarcophaga bullata (flesh flies), *C. macellaria* (blow flies) and *M. domestica* (house flies) were obtained from laboratory colonies at North Carolina State University (NCSU, Raleigh, NC, U.S.A.). *C. macellaria* was originally field collected at the NCSU poultry farm in Raleigh and has been in colony since 2012. The *S. bullata* colony originated from adults collected on the NCSU main campus in 2015. *M. domestica* was from colonies maintained at Cornell University since 1982. Rearing conditions were 25±2 °C, 50% rH and a photoperiod of 16:8 (L:D). The larval diet for house flies was a mixture of Calf-Manna (MannaPro®, Manna Pro Products, LLC, St. Louis, MO, U.S.A.), distilled water and wheat bran (The Mennel Milling CO., Fostoria, OH, U.S.A.) at a ratio of 1:7.5:15 (v/v). *C. macellaria* and *S. bullata* were reared as larvae on dry cat food (Purina Cat Chow Complete®, Nestlé Purina PetCare Company, St. Louis, MO, U.S.A.) that was soaked in water for 4 h at a ratio of 1.78:1 (v/v). Adults of all three species were fed a mixture of sugar and non-fat dry milk at a ratio of 3:1 (v/v) and supplied with tap water *ad libitum*.

Anopheles gambiae s.s (Ndokayo strain) were reared at NCSU (Deguenon *et al.*, 2020b).

The mechanical insecticide, Imergard™ WP (ImG), was provided by the company Imerys (Imerys Filtaration Minerals, Inc., Roswell, GA, U.S.A.). Imergard (https://www.imerys-performance-minerals.com/your-market/healthcare/imergard-wp) is 100% perlite active ingredient with an engineered particle size distribution to facilitate application with standard back-pack sprayers, i.e., a top cut control to prevent nozzle blocking and to optimize +ve charge to facilitate transfer of the particles from the treated surface to the mosquito.

Bioassays for median lethal time (LT_{50}) and 90% lethal time (LT_{90}) for knockdown and death

A WHO modified, cone test device was used to measure ImG activity (Fig. 1). The assay components included a glass funnel (with a 9.9 cm diameter large opening, a 1.6 cm diameter small opening and a 12 cm height), an orange stopper to prevent flies from resting in the neck of the funnel, a cap to prevent escape, and a glass Petri dish bottom (13.8 cm diameter). ImG (74.8 mg) suspended in 1 mL of absolute ethanol was added to the Petri dish bottom, which after the solvent evaporates results in a coating of 5 g ImG/m^2. The control Petri dish bottom was treated with 1 mL of ethanol only. The Petri dishes were placed in a fume hood overnight at room temperature to allow the ethanol to completely evaporate. Ethanol was used as a carrier to treat Petri dish bottoms because this method produced a reproducible

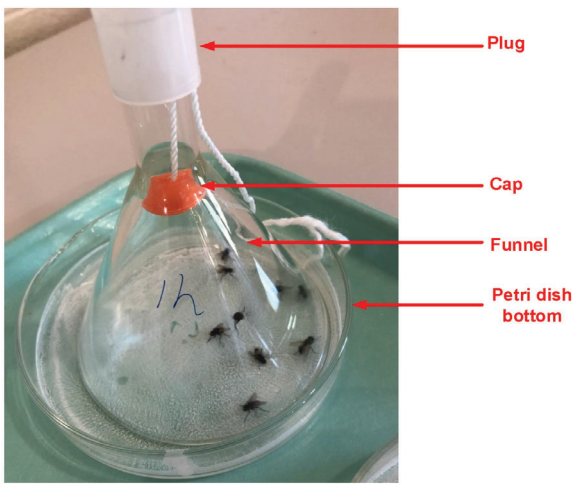


Fig. 1. Architecture for the WHO modified cone tests showing 5 g per square metre of Imergard WP on the treated glass Petri dish and house flies, *Musca domestica*, on the treated surface. See Materials and Methods for details on the cone test bioassay. The string shown is attached to the orange plug and is used to raise the plug to the sealed position after the flies are transferred to the cone test.

and reasonably uniform coverage of the MI on the plate surface (Fig. 1), was easy to set up, and did not require the use and cleanup of spray equipment. This method was shown previously to be a reliable alternative to spraying ImG in water against mosquitoes in the cone test device (Deguenon *et al.*, 2020b).

Ten 8–15-day-old adult flies (mixed ages and sex) were immobilized with carbon dioxide gas at atmospheric pressure and room temperature for 45 s for transfer using forceps to the treated and control Petri dish bottoms. They were placed on the surface ventral side down being careful not to disturb the ImG in the treatments and using the same care for control tests. Then, the cone with its plug and cap already in place was applied to the Petri dish bottom. Five replicates were conducted consisting of 3 tests per replicate where each cone test device had 10 flies. Control tests were always conducted at the same time as the treatments. The data from all three tests for each replicate were pooled to calculate the percentage mortality for that replicate. Each replicate was conducted on a different day. No cone test device was reused before cleaning. They were cleaned between tests in a dishwasher using tap water only (no soap) and heat dried by the dishwasher. Cone test devices containing insects were placed in an incubator (Model I-66NL, Percival Scientific, Inc., Perry, IA, U.S.A. incubator with temperature and humidity control) at 30± 1 °C, 50 ± 5% rH and a photoperiod of 16:8 (L:D). Humidity and temperature measurements were verified using a factory calibrated Model-971 multimeter (Everett, Washington, DC, U.S.A.). The incubator was fitted internally with an add-on light source to achieve the L:D cycle, and the same (a single) incubator was used for all bioassays in this paper. The incubation time started when the flies were placed on the Petri dish bottom after which they were transferred to the incubator in less than 5 min. Flies were considered ecologically dead when they were on their backs but still able to move. Flies were scored as dead when there was no movement when the cone test was moved quickly side-to-side a short distance (less than 1 cm). The number of ecologically dead flies and dead flies was recorded every 30 min after flies were introduced into the cone test device. Each assay ended when the mortality reached 90%, or the mortality of the control group reached 20%.

M. domestica *mortality at different application rates of ImG*

Adult house flies were chosen for the studies that follow since they are a common toxicology model to evaluate insecticides (Scott *et al.*, 2014). ImG was applied as described earlier to different cone test devices at 0.5, 1.25, 2.5, 5 and 10 g/m^2 and tests were conducted as already described. Four replicates for each rate and the control were conducted (each replicate on a different day) where each replicate was three separate cone test devices with 10 8–15-day-old flies per device.

Effect of relative humidity on M. domestica *mortality*

Ten 8–15-day-old adult house flies were transferred to each cone test device treated with 5 g/m^2 of ImG on the Petri dish bottom or ethanol alone for the controls and then incubated at 30 ± 1 °C, 16:8 (L:D), and at rHs of 30, 50, 60 and 70 ± 5%. Except for humidity differences, tests were conducted as described earlier. Three replicates consisting of three separate tests per replicate with 10 flies per test were conducted at each relative humidity for treatments and controls. The controls for each replicate were always conducted at the same time as the treatments. The data were pooled for the three tests for each replicate to calculate the response to that replicate. Replicates were conducted on different days.

Time to mortality of M. domestica *for a 3-hour exposure to ImG*

To determine the time to mortality of *M. domestica* after exposure to ImG for 3 h, 10 8–15-day-old adult house flies were placed in the cone test device with ImG at 5 g/m^2 or treated with ethanol as the control. After a 3 h exposure, the house flies were transferred to a device without ImG. Fly transfers were described earlier but in this case the carbon dioxide gas immobilization was only 10–15 s. The tests were conducted at 30 °C, 16:8 (L:D) and 50% rH. The number of ecologically dead flies and dead flies were recorded every 30 min. Three replicates consisting of three separate tests per replicate with 10 flies per test were conducted. Each replicate was conducted on a different day. The control tests were always conducted at the same time as the treatments. The data were pooled for the three tests for each replicate to calculate the response for that replicate.

Statistical analysis

Time course data and calculation of the LT_{50} and LT_{90} were conducted by Probit Analysis (PROC PROBIT, SAS V.9.4, Cary, NC, U.S.A.). Abbott's correction was performed as part of the probit procedure to correct for control mortality (Abbott, 1925). The LT_{50} and LT_{90} values were considered to be significantly different when the 95% confidence limits did not overlap.

Scanning electron microscopy

In order to detect the possible transfer of ImG from the treated surface in the modified cone test device to house flies, ImG was visualized on a house fly surface using scanning electron microscopy (SEM). Malaria mosquitoes, *Anopheles gambiae* s.s., in ImG cone test devices, were also visualized by SEM. *M. domestica* were exposed for 2 h using the MI mortality assay described earlier at the treatment rate of 5 g/m^2 and then transferred to assembled cone test devices without ImG. Immobilization with carbon dioxide gas was 10–15 s for the transfer. Devices with house flies were placed in an incubator at 30± 1 °C and 50 ± 5% rH (light-on). *An. gambiae* were transferred to devices at the ImG treatment rate of 8 g/m^2, incubated at 32 ± 1 °C and 60 ± 5% rH, and handled as described before (Deguenon *et al.*, 2020b). Flies and mosquitoes approximately 30 min before dying were transferred to 1.5 mL microcentrifuge tubes (one insect per tube) and transferred to the Analytical Instrumentation Facility at NCSU. They were mounted on

an aluminium Hitachi SEM mount with double-sided carbon tape, coated for 30 and 10 min, respectively, with 70 nm of a gold–palladium mixture (60 Au/40 Pd), and then scanned with a Hitachi S-3200 N variable pressure scanning electron microscope.

Results

The efficacy of ImG against three species of filth flies

In cone test devices (Fig. 1) to examine percentage mortality of ImG at 5 g/m^2 versus time (Fig. 2), flesh flies started dying after 3.5 h, and 90% of the flies were dead after 13 h. Death in this case was defined as no fly movement after the cone test device was shaken side to side. Blow flies started to die after 2 h with 90% mortality after a 7 h exposure. House flies began dying after 1.5 h, and 90% were dead after 8 h.

For all three fly species, flies became quiescent (knocked down), unable to fly and on their backs but still able to at least move their body parts. These flies were scored as ecologically dead. At the end of each time course (Fig. 2), the percentage mortality and the 'combined percentage dead and ecological dead' (CDED) levels approached the same value. This is suggestive that once flies were knocked down, they eventually stopped moving and were scored dead.

Probit models were generated (Table 1) for the time course data in Fig. 2, to calculate the time to 50% and 90% morality for dead flies versus the combination of dead and ecologically dead (CDED) flies. The LT_{50}s for *S. bullata*, *C. macellaria* and *M. domestica* for mortality were 7.1, 4.3 and 3.2 h, respectively; the LT_{90}s were 11.2, 7.8 and 5.7 h, respectively. The LT_{50} of *S. bullata* was significantly higher than that for *C. macellaria* and *M. domestica*, and the LT_{90} of *M. domestica* was significantly lower than that for *S. bullata*. For CDED, the LT_{50}s between the three species were significantly different, 5.5, 1.5 and 2.8 h, respectively.

M. domestica *mortality at different ImG rates*

Studies that follow were conducted on *M. domestica*. The LT_{50} and LT_{90} for different application rates of ImG in the cone test device at 30 °C and 50% rH are shown in Table 2. The LT_{50}s for death at 0.5, 1.25, 2.5, 5 and 10 g/m^2 of ImG were 8.2, 5.4, 4.7, 4.4 and 4.1 h, respectively. The LT_{90}s were 16.9, 8.4, 9.5, 6.6 and 6.4 h, respectively. For the combination of dead and ecologically dead (CDED), the LT_{50} and LT_{90} showed a similar response. The break point using death as an end point and where the shortest LT_{50} and LT_{90} occurred was the change from 0.5 to 1.25 g/m^2. Higher application rates than 1.25 g/m^2 had minor effects on time to death.

Effects of relative humidity

The LT_{50}s and LT_{90}s of ImG against *M. domestica* in cone test devices at 30, 50, 60 and 70% rH are shown in Table 3. The LT_{50}s for death were 3.4, 3.2, 4.1 and 5.5 h, respectively, and the LT_{90}s were 5.2, 5.7, 10.4 and 11.0 h, respectively. The differences in the LT_{50}s and LT_{90}s between 30 and 50% rH and between 60 and 70% rH were not significant, whereas the LT_{50}s and LT_{90}s between 50 and 70% were significantly different. In the combination of dead and ecologically dead (CDED), the LT_{50}s at 30, 50, 60 and 70% rH were 2.2, 2.8, 2.6 and 4.5 h, respectively, and for the LT_{90}s were 7.2, 6.4, 11.0 and 11.2 h, respectively.

Time to death of M. domestica *after a 3-hour exposure to ImG*

Figure 3 shows the time versus mortality against *M. domestica* for a 3-h exposure in the cone test device to ImG immediately followed by the house flies being transferred to an untreated cone test device. The test was conducted at 30 °C and 50% rH. Seven hours after the 3 h exposure to ImG, 78.33±21.67 (SEM) % of the house flies were dead, and the CDED was 83.33±16.67(SEM) %. The LT_{50}s and LT_{90}s for dead flies were 2.7 and 9.2 h, respectively, and for CDED were 2.0 and 10.2 h, respectively.

ImG on a house fly body

Figure 4 shows scanning electron micrographs of a house fly outside surface after ImG exposure in the cone test device. The micrographs show ImG on a house fly head, abdomen, thorax and wings. House fly grooming behaviour in the treatments appeared to be at a higher level than in the controls, and they were frequently on their back and circling in the treated plates with their legs moving fast. Not only their tarsi but also the whole fly body contacted the treated surface especially the thorax.

Differences in ImG coverage were found between adult house flies and mosquitoes. There were no ImG particles on house flies and mosquitoes in the controls (Fig. 5A,C, respectively). House fly and mosquito (Fig. 5B,D, respectively) lower legs and tarsi were fully covered with ImG. Deguenon *et al.* (2020b) reported that ImG particles are mostly on the mosquito lower legs. However, unlike mosquitoes, house flies were covered with high levels of ImG over the entire body (Fig. 4).

Discussion

Imergard WP demonstrated insecticidal activity against flesh flies, *S. bullata*, blow flies, *C. macellaria*, and house flies, *M. domestica*, in a modified WHO cone test device. It appears that ImG at the single application rate tested in these experiments killed blow flies and house flies faster than flesh flies (Fig. 2). Adults of a flesh fly on average weigh 77.5 mg (wet weight) as compared to blow flies and house flies at 20.1 and 15.8 mg per fly, respectively (Deguenon *et al.*, 2019a; average weight for the NC State University colonies and rearing conditions). The size (wet weight) of a flesh fly is 3.86 and 4.91 times greater than that of a blow fly and house fly, respectively. Volume increases by the cube while surface area increases by the square as the size of an

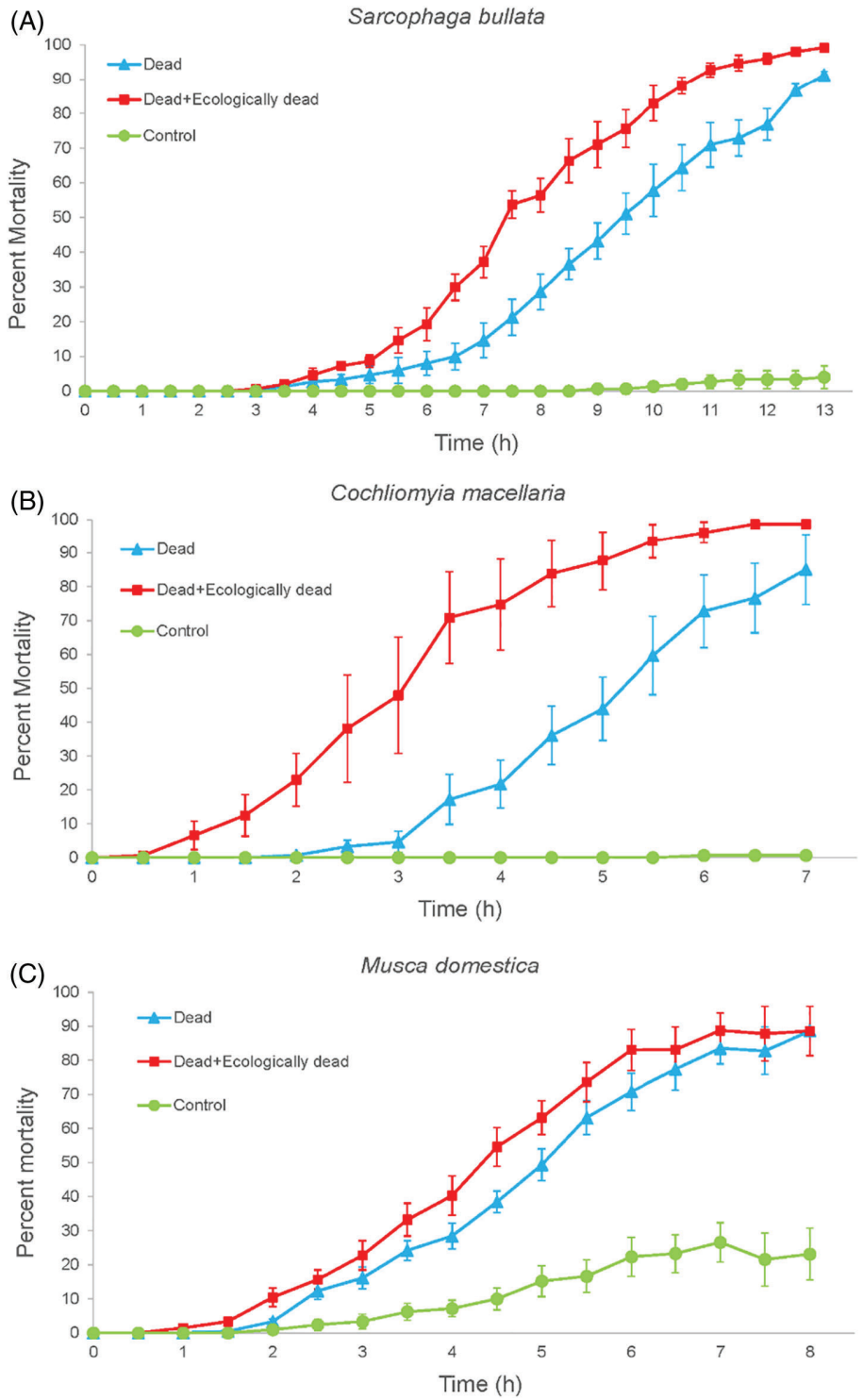


Fig. 2. Time versus percentage mortality of adult (A) flesh flies, *Sarcophaga bullata*, (B) blow flies, *Cochliomyia macellaria*, and (C) house flies, *Musca domestica* exposed to 5 g/m^2 of Imergard WP in the cone test (Fig. 1) at 30 °C and 50% rH. Insects that did not move when the cone test was moved back and forth quickly were considered dead. Insects that were on their back but still able to move were considered ecologically dead. The error bars are ±1 SEM.

Table 1. LT_{50} and LT_{90} of Imergard WP (5 g/m^2) against a flesh fly, *Sarcophaga bullata*, a blow fly, *Cochliomyia macellaria* and a house fly, *Musca domestica* in cone tests at 30 ± 1 °C and 50 ± 5% relative humidity.

Mortality	Fly species	N	Slope (SE)	LT_{50}*(95% CL†)	LT_{90}*(95% CL†)	χ^2
Dead‡	*S. bullata*	300	6.66 (0.85)	7.1A (6.61–7.73)	11.2A (9.74–14.13)	4.54
	C. macellaria	300	5.00 (0.78)	4.3B (3.85–5.13)	7.8AB (6.19–11.82)	4.80
	M. domestica	300	5.01 (0.67)	3.2C (2.91–3.53)	5.7B (4.82–7.58)	4.05
Combination of Dead and Ecologically Dead§ (CDED)	*S. bullata*	300	8.30 (0.84)	5.5A (5.23–5.74)	7.8A (7.15–8.81)	3.13
	C. macellaria	300	4.54 (0.86)	1.5B (1.32–1.78)	2.9B (2.29–4.63)	0.64
	M. domestica	300	3.61 (0.54)	2.8C (2.48–3.33)	6.4 AC (4.87–10.31)	3.76

* Values are in hours. Results were Abbott corrected.
† In each column, lethal rates with overlapping confidence limits (CL) are not significantly different and are indicated by the same letter.
‡ Insects that did not move when the cone test was moved back and forth quickly.
§ Insects that were on their back but still able to move.

Table 2. LT_{50} and LT_{90} for different rates of Imergard WP (ImG) against a house fly, *Musca domestica* in cone tests at 30 ± 1 °C and 50 ± 5% relative humidity.

Mortality	ImG (g/m^2)	n	Slope (SE)	LT_{50}*(95% CL†)	LT_{90}*(95% CL†)	χ^2
Dead‡	0.5	240	4.07 (0.51)	8.2A (7.41–9.36)	16.9A (13.69–23.77)	7.53
	1.25	240	6.84 (0.85)	5.4B (5.11–5.86)	8.4B (7.42–10.12)	4.04
	2.5	240	4.17 (0.51)	4.7BCD (4.27–5.33)	9.5B(7.76–13.21)	3.64
	5	240	7.51 (0.66)	4.4D (4.25–4.67)	6.6C (6.03–7.40)	1.87
	10	240	6.57 (0.95)	4.1CD (3.79–4.54)	6.4 BC(5.51–8.34)	3.55
Combination of Dead and Ecologically Dead§ (CDED)	0.5	240	3.15 (0.43)	7.9A (6.94–9.54)	20.2A (15.13–33.14)	8.60
	1.25	240	2.53 (0.55)	5.1AB (4.01–7.89)	16.5A (9.84–57.82)	10.93
	2.5	240	2.72 (0.48)	3.4B (2.85–4.44)	10.1A (6.84–22.27)	6.69
	5	240	4.49 (0.90)	4.2B (3.61–5.50)	8.1A (5.95–16.07)	6.22
	10	240	4.04 (1.01)	4.2AB (3.41–7.26)	8.8A (5.71–31.45)	5.42

* Values are in hours. Results were Abbott corrected.
† In each column, lethal rates with overlapping confidence limits (CL) are not significantly different and are indicated by the same letter.
‡ Insects that did not move when the cone test was moved back and forth quickly.
§ Insects that were on their back but still able to move.

Table 3. LT_{50} and LT_{90} of Imergard WP (5 g/m^2) against a house fly, *Musca domestica* in cone tests at 30± 1 °C and 30 ± 5, 50 ± 5, 60 ± 5 and 70 ± 5% relative humidity (rH).

Mortality	rH (%)	n	Slope (SE)	LT_{50}*(95% CL†)	LT_{90}* (95% CL†)	χ^2
Dead‡	30	180	7.21 (0.67)	3.4A (3.28–3.65)	5.2A (4.78–5.84)	1.79
	50	300	5.01 (0.67)	3.2A (2.91–3.53)	5.7AB (4.82–7.58)	4.05
	60	180	3.12 (0.66)	4.1AB (3.30–5.33)	10.4BC (7.16–25.00)	9.17
	70	180	4.30 (0.90)	5.5B (4.78–7.15)	11.0C (8.12–22.54)	6.84
Combination of Dead and Ecologically Dead§ (CDED)	30	180	2.54 (0.69)	2.2AB (1.64–3.91)	7.2A (4.05–50.07)	7.48
	50	300	3.61 (0.54)	2.8A (2.48–3.33)	6.4A (4.87–10.31)	3.76
	60	180	2.04 (0.39)	2.6A (1.98–3.46)	11.0A (6.78–31.27)	5.87
	70	180	3.24 (0.77)	4.5B (3.64–6.57)	11.2A (7.33–35.60)	8.65

* Values are in hours. Results were Abbott corrected.
† In each column, lethal rates with overlapping confidence limits (CL) are not significantly different and indicated by the same letter.
‡ Insects that did not move when the cone test was moved back and forth quickly.
§ Insects that were on their back but still able to move.

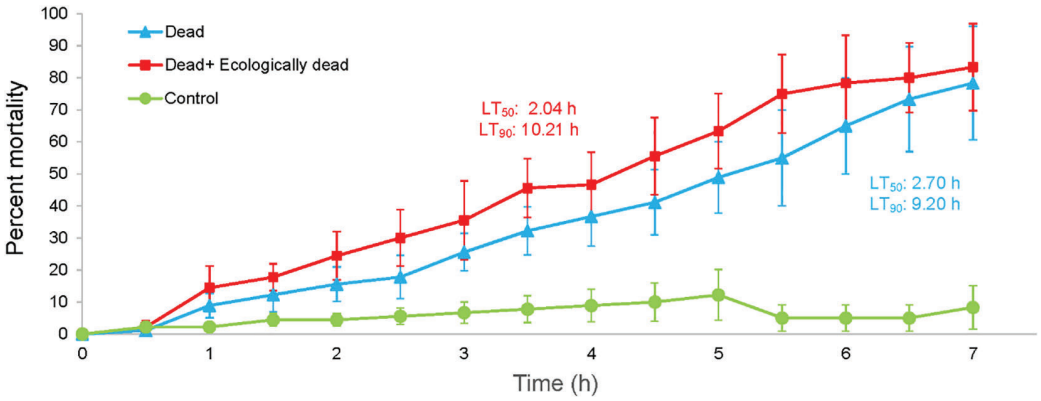


Fig. 3. Time versus percentage mortality of adult house flies, *Musca domestica*, after exposed to 5 g/m^2 of Imergard WP in the cone test (Fig. 1) at 30 °C and 50% rH for 3 h, then transferred to a cone test with no Imergard WP present and under the same environmental conditions. Insects that did not move when the cone test was moved back and forth quickly were considered dead. Insects that were on their back but still able to move were considered ecologically dead. The error bars are ±1 SEM.

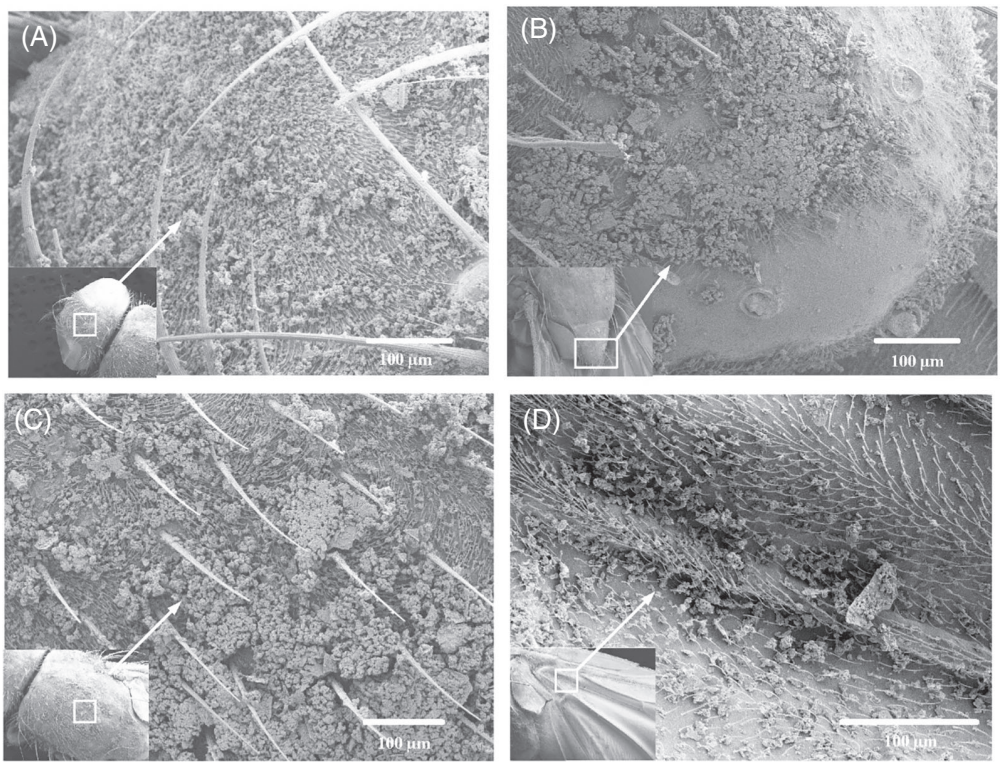


Fig. 4. Scanning electron micrographs of adults of a house fly, *Musca domestica*, exposed to Imergard WP in the cone test (Fig. 1). *M. domestica* were transferred to cones for 2 h at the treatment rate of 5 g/m^2 and then transferred to assembled cones without ImG. Cones were placed in an incubator at 30± 1 °C and 50 ± 5% rH. Flies within 30 min of dying were transferred to 1.5 mL microcentrifuge tubes (1 fly per tube) for imaging. (A) Head, (B) abdomen, (C) thorax, (D) wing. Inserted pictures in the left bottom corner show the area of each body part (indicated by the arrow) in greater magnification.

insect increases and where the general insect shape is similar between comparative species. The surface area to volume ratio of a fly with a larger body size then is relatively small compared to flies with a smaller body size (Kühsel *et al.*, 2017). Thus, it would be predicted that flesh flies would be less susceptible than blow flies and house flies to a topically active insecticide based on their greater weight and smaller surface area to volume ratio. Our research here supports this hypothesis. Dawson (2004) also reported for diatomaceous earth (DE), the activity was higher for a smaller body size.

Desiccation tolerance also may affect susceptibility to MIs (Guedes *et al.*, 2015). Korunic (1998) reported that DE was more

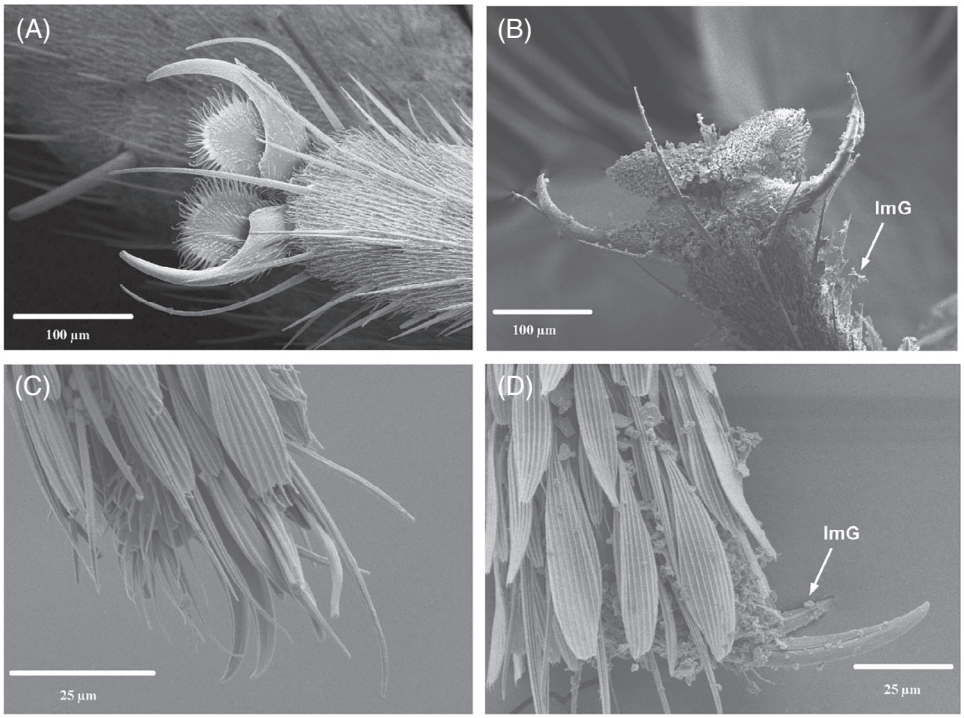


Fig. 5. Scanning electron micrographs of the tarsi of an adult house fly, *Musca domestica*, compared to that of the malaria mosquito, *Anopheles gambiae*. *M. domestica* were transferred to cones for 2 h at the treatment rate of 5 g/m^2 and then transferred to assembled cones without ImG. Cones with house flies were placed in an incubator at 30 ± 1 °C and 50 ± 5% rH. *An. gambiae* were transferred to cones at the ImG treatment rate of 8 g/m^2 and placed in an incubator at 32 ± 1 °C and 60 ± 5% rH. Flies and mosquitoes within 30 min of dying were transferred to 1.5 mL microcentrifuge tubes (1 test insect per tube) for imaging. (A) *Musca domestica* untreated tarsi, (B) *Musca domestica* ImG treated tarsi, (C) *Anopheles gambiae* untreated tarsi, (D) *Anopheles gambiae* ImG treated tarsi.

effective against true bugs that had setaceous and rough surfaces. The assumption was that the 'hairy' surface provided more area for evaporation and collection of the MI leading to greater cuticle damage and water evaporation. The reduced susceptibility of a flesh fly to ImG could be the result of a low surface to volume ratio, differences in setae density and/or other unknown factors. More research is needed with ImG to evaluate these factors.

A modified WHO cone test device was used to determine the impact of the application rate on the efficacy of ImG against house flies. The authors found that application rates higher than 1.25 g/m^2 had a minor impact on shortening the time to death (Table 2). Collins & Cook (2006) also reported that a low rate of 5 g/m^2 for DE was as effective as a high rate of 20 g/m^2. By observation, the amount of ImG remaining on the cone test device bottom at the end of the test was different at different treatment rates. For example, the plates treated with 0.5 g/m^2 of ImG at the end of the experiment were similar visually to the control assay where no ImG was applied; the flies transferred almost all the MI to their bodies. The amount of residual ImG on the plate at the end of each experiment then increased as the treatment rate increased. Since the total fly surface area is finite, once the flies have saturated their surface with ImG, increasing the amount of the MI per unit area on the treated surface of the cone test device will have a minimal or no impact on time to death. In the field, however, the residual activity effective in killing flies after an application is also an important factor, essentially 'the longer the better' (excluding other concerns like the evolution of resistance or potential non-target effects). For this reason, the authors tested ImG applied at the rate of 8 g/m^2 to interior hut walls and the ceiling of experimental huts in Africa for mosquito control; this rate achieved at least 6 months of control (Deguenon *et al.*, 2020a). The same principle could apply for filth fly control, the higher the treatment rate, the longer the duration of control. More research is needed in the field to optimize the level of control versus the duration of control for ImG for both mosquitoes and filth flies.

The mortality rate of *M. domestica* significantly (statistically) decreased as relative humidity increased (Table 3). This result was consistent with studies on DE. Athanassiou *et al.* (2007) found that the effectiveness of DE against the larger grain borer, *Prostephanus truncatus*, decreased as the relative humidity increased from 55% to 75% at three temperatures. Korunic (1998) reported similar findings for the red flour beetle, *Tribolium castaneum*, especially at a humidity above 70%. The same trend between humidity and mortality was also reported for other MIs. Arthur & Puterka (2002) reported that more insects survived at a relative humidity of 57% and 75% than 40% when exposed to Kaolin-based particle films. Zeolites were also reported to be more effective as an insecticide at a low and intermediate relative humidity compared to high humidity (Floros *et al.*, 2018). Stadler *et al.* (2012) found that to achieve the same 50% mortality for two different stored grain pests at

80% ambient humidity, the rate of a nanostructured alumina had to be twice that of a low rate. The efficiency of ImG also decreased at the high relative humidity tested in our studies, which suggested the mode of action was desiccation. The rate of water loss from the insect is reduced as the humidity increases, which reduces the mortality rate of the mechanical insecticide (Mewis & Ulrichs, 2001). Cribb *et al.* (2013) suggested this inverse relationship between humidity levels and mortality occurs because DE adsorbs water from the air, which reduces its capacity to adsorb lipids from the insect's cuticle. However, ImG is not hygroscopic (personal communication, David Stewart). Another possibility not mentioned before for an MI, this relationship between decreased efficacy with increased humidity for ImG and other MIs could be decreased static transfer from a treated surface to the insect cuticle. This is especially relevant for mosquitoes discussed in more detail later.

M. domestica were exposed to ImG for 3 h at 30 °C and 50% rH in the cone test device, and then transferred to untreated devices held under the same environmental conditions. Delayed mortality was observed (Fig. 3) after the transfer. More studies will be needed in the laboratory and the field to better understand the time of exposure to a treated surface versus percentage knockdown or mortality. At least after a 3 h exposure in the cone test, ImG was lethal. In field studies with mosquitoes under practical field conditions where the insects could make choices between treated and untreated surfaces or even have access to hydration, high levels of control were found similar or greater than the chemical insecticide, positive controls (Deguenon *et al.*, 2020a, 2020b). It remains to be seen if this will be the case for filth flies.

Deguenon *et al.* (2020a, 2020b) found that mosquitoes after landing on a treated surface in an ImG cone test became quiescent. Irrespective of this, the physical interactions between the mosquito and the treated surface by observation were at a much lower shear force than flies and mostly limited to landing and standing on their tarsi. In mosquitoes, SEM studies showed most of the ImG was on the lower legs as shown also in this current study (Fig. 5D). House fly interactions with the treated surface were quite different from mosquitoes. By observation, they constantly flew into the treated bottom surface of the cone test device, walked around the surface more than mosquitoes, and appeared to increase their overall activity including grooming. Although they showed similar levels of ImG on their lower legs as mosquitoes (Fig. 5B), house flies had much higher levels on the other parts of their body (Fig. 4) than that shown by Deguenon *et al.* (2020b) for mosquitoes. From observations by SEM with comparisons to mosquitoes, the amount of ImG coverage on the outside total surface of a house fly appeared to be much greater than mosquitoes. Exactly how the surface became covered like this for a house fly is not certain, but likely a result of the high force interactions with the treated surface from flight, the constant walking on the surface, fly–fly interactions and grooming, which was not observed in mosquitoes. In mosquitoes, it appears ImG on the body was by static transfer from the treated surface. Since the authors do not understand the site of action on the insect of ImG that causes death, the widespread coverage of ImG on the fly surface may have no significance relative to mortality. Mosquitoes died in about the same amount of time but in this case the ImG was mostly on their lower legs. The exact amount of ImG needed on the insect surface to cause death is also unknown at this juncture. However, considering that only a small amount of ImG was on the lower legs of the mosquito and this resulted in death, this raises the possibility that the additional coverage over a house fly body may not be needed to kill the insect and much lower levels are needed for death than what appears on the total outside surface of the fly.

The mode of action of MIs is generally thought to be a physical action that damages the insect's cuticle by abrasion and/or lipid absorption resulting in disruption of water balance (Korunić *et al.*, 2016). Deguenon *et al.* (2020a, 2020b) suggested that the mode of action of ImG against mosquitoes might not be a physical disruption of their cuticle by abrasion since mosquitoes are not active once they make contact with the treated surface in the cone test device and do not appear to be grooming. In the present study, the authors cannot exclude abrasion as a factor in house fly death since large amounts of ImG were found over the whole body and the flies were hyperkinetic. Mortality of *M. domestica* significantly decreased as relative humidity increased (Table 3), suggesting the mode of action was disruption of water balance.

This paper is the first study of the use of ImG against filth flies (*S. bullata*, *C. macellaria* and *M. domestica*) and the first study of the impact of application rate of ImG on insect mortality. Our results suggest that ImG could be an alternative method for filth fly control. Perlite, the active ingredient of ImG, is generally recognized as a safe substance for other uses (Perlite Institute, 2020). ImG for insect control can be applied in water with standard spray equipment used in vector control and provides long-term control; for mosquitoes in experimental huts in Africa, 80% of control was obtained for 6 months (Deguenon *et al.*, 2020a). Since ImG is an industrial mineral, it does not degrade from abiotic factors like high heat or UV light. Imergard also does not contain crystalline silica. Additional work is needed like already conducted for mosquitoes to test the efficacy of ImG under practical conditions for filth fly control (for example, treating vertical surfaces).

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Author contributions

Conceptualization, KC, JMD, MHR, DWW, DAS, DG, CSM and RMR; Methodology, KC, JMD, GC, SSD, MHR, DWW, DAS, DG, CSM and RMR; Validation, KC, JMD, GC, MHR, DWW, DAS, DG, YZ, XL, CSM and RMR; Investigation, KC, JMD, GC, SSD, DAS and CSM; Resources, SSD, MHR, DWW, DAS, DG, YZ, XL and RMR; Writing-Original Draft, KC and RMR; Writing-Review & Editing, KC, JMD, GC, SSD, MHR, DWW, DAS, DG, YZ, XL, CSM and RMR; Supervision, KC, JMD, MHR, DWW, DAS, DG, YZ, XL, CSM and RMR; Project Administration, KC, JMD, SSD, MHR, DWW, DAS, CSM and RMR; Funding Acquistion, JMD, MHR, DWW, DAS, DG, YZ, XL and RMR.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Short Communication

Short Communication

New Mosquitocide Derived From Volcanic Rock

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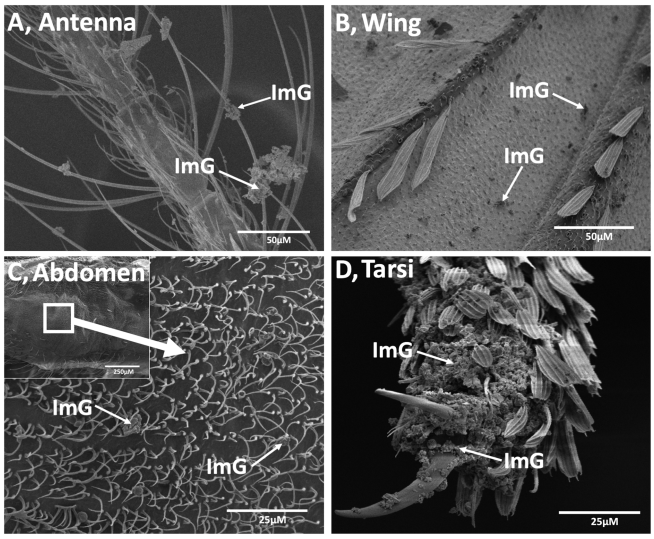
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Abstract

Malaria, dengue, yellow fever, and the Zika and West Nile Viruses are major vector-borne diseases of humans transmitted by mosquitoes. According to the World Health Organization, over 80% of the world's population is at risk of contacting these diseases. Insecticides are critical for mosquito control and disease prevention, and insect insecticide resistance is on the increase; new alternatives with potentially different modes of action from current chemistry are needed. During laboratory screening of industrial minerals for insecticide activity against *Anopheles gambiae* (Giles) (Diptera: Culicidae) we discovered a novel mechanical insecticide derived from volcanic rock (MI_{VR}) with potential use as a residual spray. In modified WHO cone tests, the time to 50% mortality was 5 h under high-humidity conditions. MI_{VR} treated surfaces demonstrated no mosquito repellency. In field studies where the mechanical insecticide was applied to wood using standard spray equipment and then placed under stilt homes in New Orleans, LA, the residual activity was >80% after 9 wk against *Aedes aegypti* (L.) (Diptera: Culicidae), *Aedes albopictus* (Skuse) (Diptera: Culicidae) and *Culex quinquefasciatus* (Say) (Diptera: Culicidae) (with similar efficacy to a positive chemical insecticide control). In scanning electron microcopy studies, the MI_{VR} was transferred as particles mostly to the legs of the mosquito. This wettable powder made from volcanic rock is a mechanical insecticide representing a potential new mode of action different from current chemistry for mosquito control and is in commercial development under the trade name Imergard™ WP as an indoor and outdoor residual spray.

Graphical Abstract



Key words: Imergard™WP, mechanical insecticide, mosquito-borne disease, mosquitoes, residual spray

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Mosquitoes are among the deadliest animals in the world (WHO 2020a). The protozoan that causes Malaria is transmitted by anopheline mosquitoes with over 200 million cases and 400,000 deaths every year, especially in developing countries. In addition, over 3.9 billion people in >129 countries are at risk for Dengue, a mosquito-borne viral infection transmitted by *Aedes* mosquitoes. Also, the West Nile virus transmitted by *Culex* mosquitoes is now endemic in >100 countries including the United States (Konongoi et al. 2016, Hadfield et al. 2019, WHO 2020a).

Mosquito control constitutes the main approach for disease prevention (WHO 2020a). This is mainly achieved by the use of chemical insecticides with systemic modes of action to reduce vector populations and vector–human contact; this includes insecticide-treated bed nets, personal insect repellents, larvicides, and indoor and outdoor residual spraying (Curtis et al. 2006, Frances 2007, Smith et al. 2016, Sherrard-Smith et al. 2018, Kaur et al. 2019).

The prolonged use of chemical insecticides is often associated with negative impacts on the environment and human health and the development of insecticide resistance (Liu and Yue 2000, Eskenazi et al. 2018, Deguenon et al. 2019). Mosquito resistance is now widespread for the pyrethroids, the most commonly used insecticide in vector control, but also for other pesticide classes like the carbamates, organophosphates, and organochlorines (Aïkpon et al. 2014, Moyes et al. 2017). New actives with different modes of action from current chemistry and potential minimal environmental impact are urgently needed for mosquito control.

We report here the discovery of a mechanical insecticide (MI) derived from 100% expanded Perlite. Perlite is an aluminosilicate volcanic rock (VR) categorized by the US Food and Drug Administration (FDA) as 'Generally Recognized as Safe (GRAS)' and classified by the European Food Safety Authority (EFSA) as an animal feed additive (Maxim et al. 2014). Mechanical insecticide derived from volcanic rock (MI_{VR}) has never been used before in vector control and provides a new, nonsystemic, mechanical mode of action certainly different from current chemistry for mosquito control, likely affecting water balance based on what is known about other mechanical insecticides (Ebeling and Wagner 1959, Lockey 1988).

Materials and Methods

Mosquitocidal Activity Determined Using Cone Tests

Anopheles gambiae s.s. (Ndokayo strain, Giles, Diptera: Culicidae) used in cone tests were reared in the Dearstyne Entomology laboratory at North Carolina State University (NCSU) using procedures described by Benedict (2007). The eggs were obtained through BEI Resources, NIAID, NIH from a colony that originated in 2008 in the village Ndokayo (5°30.723′ N 14°07.497′ E), located in eastern Cameroon (https://www.beiresources.org/Catalog/BEIVectors/MRA-1278.aspx) and maintained under laboratory conditions with no insecticide selection pressure.

The MI_{VR} was provided by the company Imerys (Imerys Filtration Minerals, Inc., Roswell, GA). Perlite is the active ingredient for the mechanical insecticide made from VR. The VR is expanded with heat (760–980°C) and then processed by drying and grinding to obtain a fine white powder (MI_{VR} = Imergard™WP, Fig. 1A). In the studies that follow, the MI_{VR} is applied as a suspension in absolute ethanol for cone tests or by spraying in water (described later).

Cone tests were comprised of a glass funnel (large opening = 9.9 cm; small opening = 1.6 cm; and height = 12 cm), a stopper to prevent mosquitoes from resting in the neck of the funnel, a cap to prevent escape, and a glass Petri dish bottom (diameter = 11.8 cm, Fig. 1B). Preliminary tests showed 1 ml of an absolute ethanol carrier used in these studies was enough to completely cover the glass Petri dish bottom. A MI_{VR} suspension in 1 ml of ethanol was chosen to produce a final concentration of 8 g of MI per square meter, when allowed to dry on the Petri dish bottom. The control received 1 ml of ethanol only. The ethanol was allowed to air dry at room temperature and ambient humidity for 24 h before use. Twenty-five, nonblood-fed, 2- to 5-d-old female mosquitoes were introduced into each cone using a mouth aspirator (Model: 612, John W. Hock Company, Gainesville, FL). The assays were conducted at 32 ± 1°C and 60 ± 5% relative humidity. Mortality was recorded every hour until ≥80% of the mosquitoes were dead (i.e., laying on their back with no movement after gently shaking the cone). The tests were conducted in triplicate.

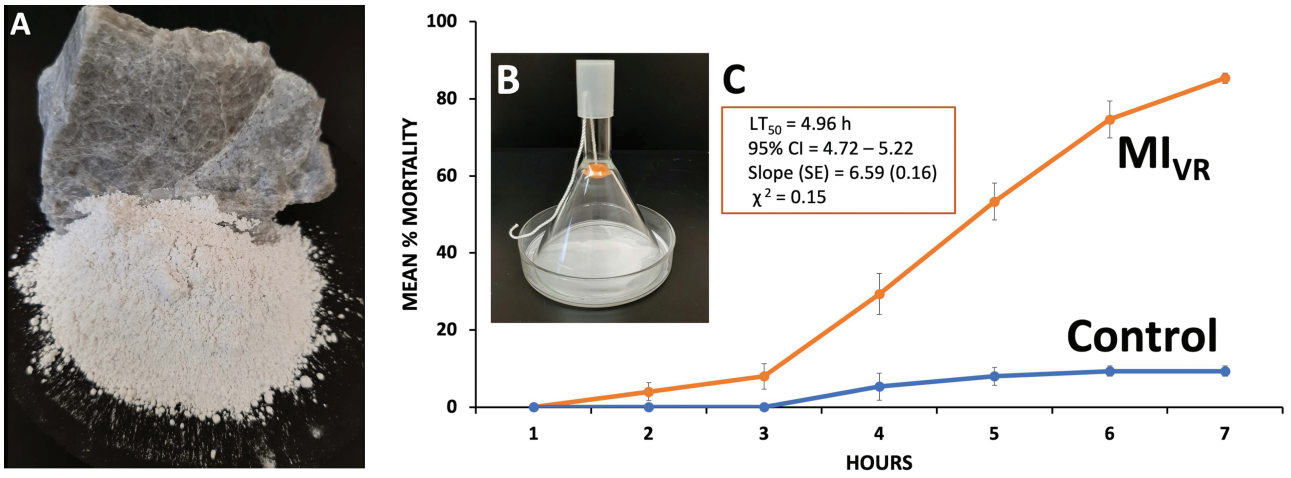


Fig. 1. (A) Perlite is the active ingredient for the MI made from VR (top) that can be expanded (when heated to 760–980°C) and then processed (by drying and grinding) to obtain the fine white powder (MI_{VR} = Imergard™ WP, bottom), (B) is a modified WHO cone test used for testing the MI_{VR} (coated on the bottom of the Petri dish) efficacy against adults of *Anopheles gamibae* and (C) is time versus mortality for the MI_{VR} versus a negative control with Probit analysis (LT_{50} = time for 50% mortality [Abbott corrected]). SE = standard error; CI = confidence interval.

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Repellency Studies

To determine if mosquitoes would be repelled from a MI_{VR} treated surface, a two-choice bioassay was performed by methods described by Bissinger et al. (2008, 2009) using the funnel setup as described earlier (Fig. 1B). For repellency assays, however, one half of the glass Petri dish bottom was covered with adhesive tape, and then the plate treated as described before to obtain 8 g of MI/m^2. After drying, the tape was removed leaving an untreated surface that was then covered with a half circle of Whatman No. 2 filter paper (Whatman PLC, Maidstone, United Kingdom). The filter paper was treated with 0.5 ml of ethanol only and dried overnight before being added to the Petri dish. The glass funnel was inverted onto the Petri dish bottom, 10 adult female mosquitoes introduced into the inverted funnel as described before, and the assay allowed to run for 30 min with no disturbance. At the end, the number of mosquitoes and their position on the arena floor on either side of the Petri dish bottom was recorded. The bioassays were conducted at 25°C and 55% relative humidity under ambient laboratory lighting. Trials were replicated six times. A control with two ethanol-treated filter paper semicircles was also conducted to account for any unknown external environmental cues that might have affected mosquito positioning.

Scanning Electron Microscopy

In order to determine the possible transfer of the MI_{VR} from the treated surface (Fig. 1B) to the mosquito, the mechanical insecticide was visualized on the mosquito using scanning electron microscopy (SEM). *Anopheles gambiae* were exposed using the MI mortality assay described earlier. Mosquitoes within 30 min of dying were transferred to 1.5-ml microcentrifuge tubes (one mosquito per tube) and transferred to the Analytical Instrumentation Facility at NCSU for imaging. There was no evidence by visual inspection that the MI was transferring to the inner surface of the tubes. The mosquitoes were mounted on an aluminum Hitachi SEM mount with double-sided carbon tape, coated for 10 min with ~70 nm of a gold-palladium mixture (60 Au/40 Pd), and then scanned with a Hitachi S-3200N variable pressure scanning electron microscope.

Field Stability Studies of MI Sprayed in Water on Wood and Tested Against Additional Mosquito Species

To better understand the potential use of the MI_{VR} (=Imergard™WP) for vector control and its residual activity under field conditions, a 9-wk field trial was conducted by the New Orleans Mosquito, Termite and Rodent Control Board, in New Orleans, LA. Eighteen properties (stilt houses) were selected based on their housing structures (raised foundation), location (suburban), surrounding backyard vegetation (green and lush), and presence of mosquito breeding sites (water holding containers). Wood chips cut from untreated No. 2 yellow pine (5.1 × 10.2 cm boards) obtained from a local hardware store (used for home construction in the United States) were treated and placed in a metal tray under each house. Wood chips were used as an alternative substrate to that of the glass surface investigated in our laboratory studies (described earlier) and more representative of a field application situation. The houses were divided into three sites as follows: six houses had the MI_{VR}-treated wood chips, six received lambda-cyhalothrin treated wood chips (Demand CS, 9.7% active ingredient/liter, Syngenta Crop Protection, Greensboro, NC; a pyrethroid used as a positive control) and six received water treated wood chips as a negative control. The MI was applied by spraying in water at a rate of 5 g/m^2. The pyrethroid was applied at a rate of 20 mg/m^2. One wood chip was transferred to the laboratory each week for weeks 3–9 to measure residual activity.

The wooden substrate was placed in a separate bag from each test condition in the field, labeled appropriately and transferred to the New Orleans Mosquito, Termite and Rodent Control Board Laboratory (New Orleans, LA). The wood chips were placed into separate 355-ml paper cups (Schneider Paper Products, Inc. New Orleans, LA) with mesh lids to prevent escape and mosquitoes transferred to each cup to interact with the treated wood. Five laboratory reared female mosquitoes (2- to 5-d old) were transferred to each cup using an aspirator (model 13500, Clarke, St. Charles, IL). A cotton ball soaked in 10% sugar solution was placed on top of each mesh lid. Incubations were conducted at 27°C and 75% relative humidity with mortality (defined earlier) recorded after 24 h. Three mosquito species were tested in the study: the yellow fever mosquito, *Aedes aegypti* (L., Diptera: Culicidae), the Asian tiger mosquito, *Aedes albopictus* (Skuse, Diptera: Culicidae), and the West Nile virus mosquito, *Culex quinquefasciatus* (Say, Diptera: Culicidae). Meteorological data (temperature, humidity, and precipitation) was collected using Weather Underground (www.wunderground.com; 2016, The Weather Company, LLC).

Statistical Analysis

The raw data were managed using Microsoft Excel (Version 2016). Time-mortality data from cone tests were subjected to logistic regression analysis by Probit (Finney 1971). Abbott's transformation was performed as part of the probit procedure to correct for control mortality (Abbott 1925). In the repellency tests, proportions of mosquitoes found on each side of the Petri dish bottom were calculated (number of mosquitoes found on either side divided by the total number mosquitoes found on both sides). A Wilcoxon signed-rank test was then performed to determine statistical differences. For the New Orleans study, a Mann–Whitney *U*-test was performed between Imergard™WP and lambda-cyhalothrin using weeks as replicates. All statistical analyses were performed using the R statistical software (R Foundation for Statistical Computing, Vienna, Austria; R Development Core Team 2016).

Results

New Mosquitocide from VR

Our research describes the discovery of mosquitocidal activity for a novel MI (Fig. 1A) derived from VR. A modified WHO cone test (Fig. 1B) was used to measure the insecticidal activity of the MI_{VR} (Fig. 1A) as a potential residual spray against insecticide-susceptible adults of the African malaria mosquito, *An. gambiae* (*s.s.*). In our screening, the MI_{VR} was surprisingly efficacious as a mosquitocide (Fig. 1C). The first mortality occurred above that of the control in as little as 2 h and increased with time. The time to 50% mortality (the LT_{50}, Abbot corrected) was 4.96 h with >80% mortality at 7 h.

Behavioral assays were conducted to determine whether mosquitoes would avoid a surface treated with the MI_{VR}. The methods were modified from a two-choice assay previously developed in our laboratory (Bissinger et al. 2008, 2009). In assays with two untreated sides, the percentage of mosquitoes found on the whole Petri dish bottom was 83.3 ± 4.9 (SE); the percentage on the whole Petri dish bottom for MI_{VR} versus the untreated choice was 78.3 ± 4.0. The mosquitoes not on the Petri dish bottom were on the inner surface of the glass cone.

No significant difference was found in the proportion of mosquitoes found on the MI-treated surface versus the untreated control

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side (Wilcoxon signed-rank test, $V = 11$, $P = 0.407$). The median proportions of mosquitoes were 55.6 versus 44.4%. When given a choice between two untreated sides, no significant difference (Wilcoxon signed-rank test, $V = 2.5$, $P = 0.423$) was found between the proportion of mosquitoes on the left (47.2%) versus the right (52.8%) sides of the Petri dish bottom; these results demonstrate that the mosquito distribution was not driven by any unknown external cues. A schematic of the position of each mosquito on the Petri dish surface is shown with open circles for all replicates in these tests (Fig. 2). The MI_{VR} was not repellent to the mosquitoes tested, which is critical for the use of this mineral as a residual spray application. The MI developed from VR was accepted for commercial development under the trade name, Imergard™WP (ImG).

ImergardWP on the Mosquito Surface

Figure 3 shows scanning electron micrographs (SEMs) of the mosquito's outside surface after ImG exposure in the cone test (Fig. 1B). Mosquitoes come in contact with the treated surface in the cone test by landing and standing on their tarsi. No grooming behavior was observed, and there was no direct contact of the mosquito body with the treated surface until knock down and death. The mosquitoes become quiescent, eventually are knocked down (on their sides and back but still moving) and then die. Mortality is defined as mosquitoes that are knocked down and do not move when the cone is gently shaken. The SEMs (Fig. 3) show a lethal dose of ImG on the outside of the mosquito within 30 min after death. Figure 3A–D shows the presence of the MI on the antennae, wings, abdomen, and tarsi, respectively. Interestingly, the area of highest concentration of the MI_{VR} is on the mosquito leg, especially the tarsi. As shown in Fig. 3B and C, there were no visible perturbations in the cuticle in the area where the MI came into contact with the insect surface.

Field Trials in the United States

Since significant mosquito insecticidal activity was found using a modified WHO cone test (Fig. 1) and a treated surface was not repellent to the insects (Fig. 2), a field study was conducted to evaluate the ImG residual activity under more practical conditions in an area of the United States known for high-humidity conditions and significant mosquito problems. We also examined an application on wood (a different substrate than that used in the laboratory), examined activity using a spray application in water, and evaluated the MI against different mosquito species from what was used in laboratory studies. The research was conducted by an independent contractor using their experimental design to eliminate any potential bias.

Activity of ImG after the wood chips were placed under stilt houses (Fig. 4A) was compared with a synthetic pyrethroid positive and a negative control in a 24-h mortality assay for three adult mosquito species. Figure 4B shows the percentage control each week for each treatment and the temperature, humidity, and rain fall during the study. No mortality was observed for the negative water control (Fig. 4B). For *Ae. aegypti*, the yellow fever mosquito, the median percent mortality for both Imergard WP and the pyrethroid, lambda-cyhalothrin (the positive control) was 100% (Fig. 4B) with no significant difference in control between three and nine weeks (Mann–Whitney *U*-test, $W = 17.5$, $P = 0.173$; Fig. 4C). For *Ae. albopictus*, the median percent mortality for Imergard™WP was 90% and for lambda-cyhalothrin 100% for weeks 3–9 with a statistically significant difference between the two treatments (Mann–Whitney *U*-test, $W = 7$, $P = 0.011$; Fig. 4C). For *Cx. quinquefasciatus*, the mortality was 93 % for Imergard™WP and 100 % for lambda-cyhalothrin (Fig. 4B and C; Mann–Whitney *U*-test, $W = 6.5$, $P = 0.017$). During this study, the outside temperature varied between 12 and 27°C (Fig. 4B) with an average of 20.3 ± 1.9°C (Fig. 4C) and a relative humidity between 58 and 95% (Fig. 4B) with an average of 73.6 ± 4.5% (Fig. 4C). The only rainfall was during week 5, 7 mm (Fig. 4B). These studies suggested that the MI_{VR} had a similar residual activity and in one case the same mosquitocidal efficacy as a commonly used pyrethroid insecticide used in vector control.

Discussion

Malaria, dengue, yellow fever, and Zika are diseases transmitted by mosquitoes that are of global significance to human health (WHO 2017). According to the WHO, over 80% of the world's population is at risk from at least one major vector-borne disease (WHO 2017).

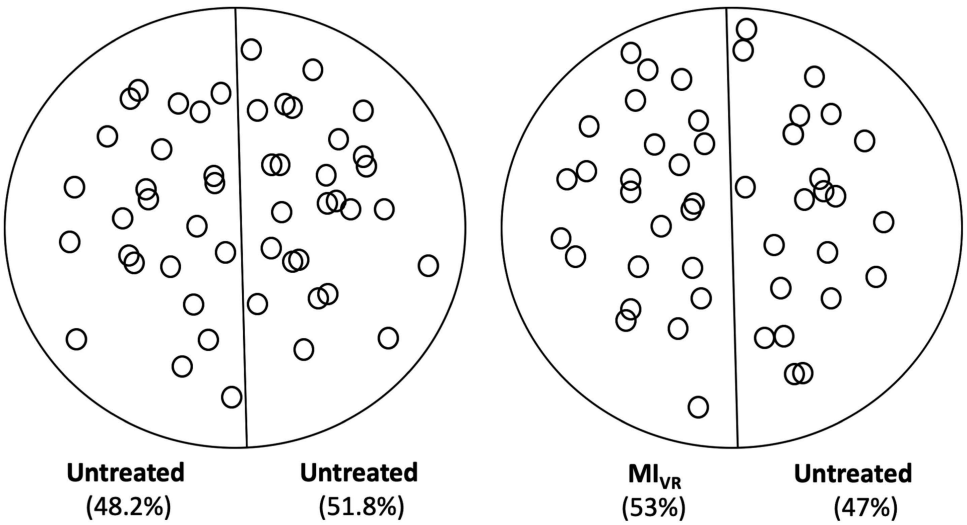


Fig. 2. Total distribution of *Anopheles gambiae* adults in repellency tests ($n = 6$) with the circles showing the position of each mosquito at 30 min. These choice studies were conducted using the cone bioassay (Fig. 1B) where one side was coated with the MI_{VR} and the other half of the petri dish bottom was untreated and covered with white filter paper (MI_{VR} versus untreated, Fig. 2) with an untreated control as shown. The percentage values in parentheses represent the overall proportions of mosquitoes found on the Petri dish bottom during the study.

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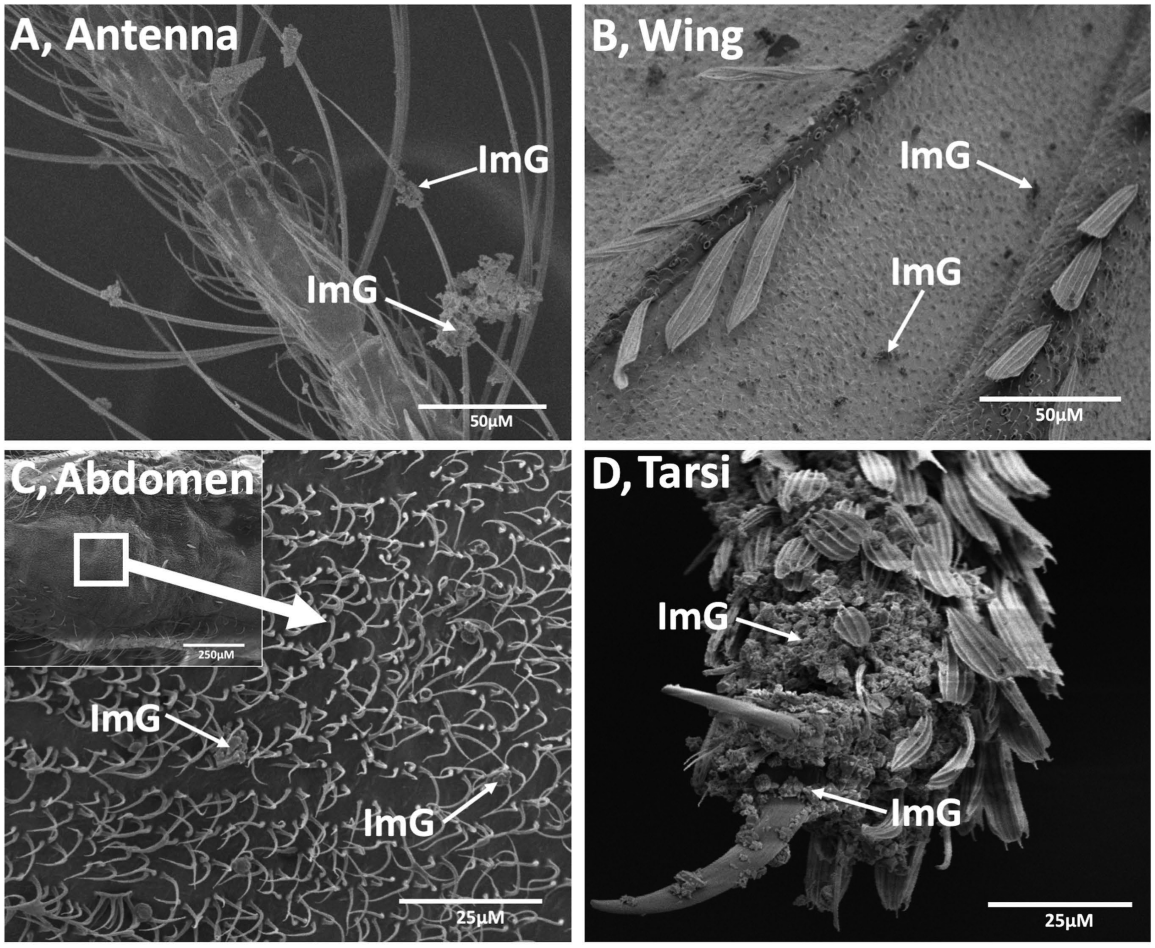


Fig. 3. Scanning electron micrographs of *Anopheles gambiae* adults exposed to MI_{VR} in the cone test (Fig. 1B) and receiving a lethal dose. (A) antenna (750×), (B) wing (750×), (C) abdomen (1,500×), and (D) lower leg (tarsi) (1,500×). Box in Fig. 3C shows the area of the abdomen shown in a greater magnification at the end of the arrow. Arrows point to different particles of Imergard™ WP on the insect surface. Control SEMs do not show any other material on the mosquito cuticle. ImG = Imergard™ WP.

The development of mosquito resistance to chemical insecticides used in vector control (Aïkpon et al. 2014, Moyes et al. 2017) and the potential negative impacts of the long-term use of these pesticides on the environment calls for the development of new and safer actives.

Proof-of-concept laboratory studies were conducted on a MI_{VR} as a residual spray for mosquito control. In modified WHO cone tests, the mechanical insecticide applied to a solid surface (a Petri dish) demonstrated time-dependent mortality against *An. gambiae* adults in as little as 2 h, with a time to 50% mortality of 5 h and greater than 80% mortality in 7 h. This activity occurred under high humidity conditions of 60% relative humidity. These studies were conducted under high humidity since these conditions are typical of places where mosquitoes are found in high density. Furthermore, there is a general thinking that mechanical insecticides like diatomaceous earth are not effective in high humidity.

The MI_{VR} as a residual spray would be optimal if the treated surface does not repel mosquitoes. In repellency studies, there was no difference in the mosquito distribution on the flat surface of the cone test between the treated and untreated side. This suggests in real-world applications like indoor residual wall sprays in Africa, community-level insect control would not be affected by mosquitoes avoiding homes treated with Imergard™WP. There is now widespread mosquito resistance to most vector control chemistries in Africa (Aïkpon et al. 2014), and the resistant mosquitoes are repelled from treated surfaces (Agossa et al. 2014).

Field studies were conducted in New Orleans for further proof of concept to i) better understand the residual activity of our mechanical insecticide in real-world and under high-humidity conditions, ii) to evaluate its performance on a different substrate (wood) after application by spraying in water, and iii) to examine its residual activity against additional mosquito species. The MI_{VR} demonstrated high mortality rates 3–9 wk after treatment (similar to that of the positive, chemical insecticide control, lambda-cyhalothrin), against three additional mosquito species, *Ae. aegypti* (median 100% mortality), *Ae albopictus* (90%), and *Cx. quinquefasciatus* (93%). These results in toto supported the decision for further commercial development of a new mechanical insecticide made from volcanic rock, Imergard™WP.

It is generally thought that mechanical insecticides transfer to the insect cuticle and disrupt the lipid layer by abrasion and/or lipid absorption (Ebeling and Wagner 1959, Lockey 1988, Ulrichs et al. 2006). In our mortality assays, it appears that the mosquitoes land on a treated surface, become quiescent (are not grooming or rolling onto the treated surface), and then start dying at 2 h. Scanning electron microscopy studies were conducted to visualize the MI on the mosquito surface. The SEM studies show that most of the MI as particles are on the legs, especially the tarsi and much less on the rest

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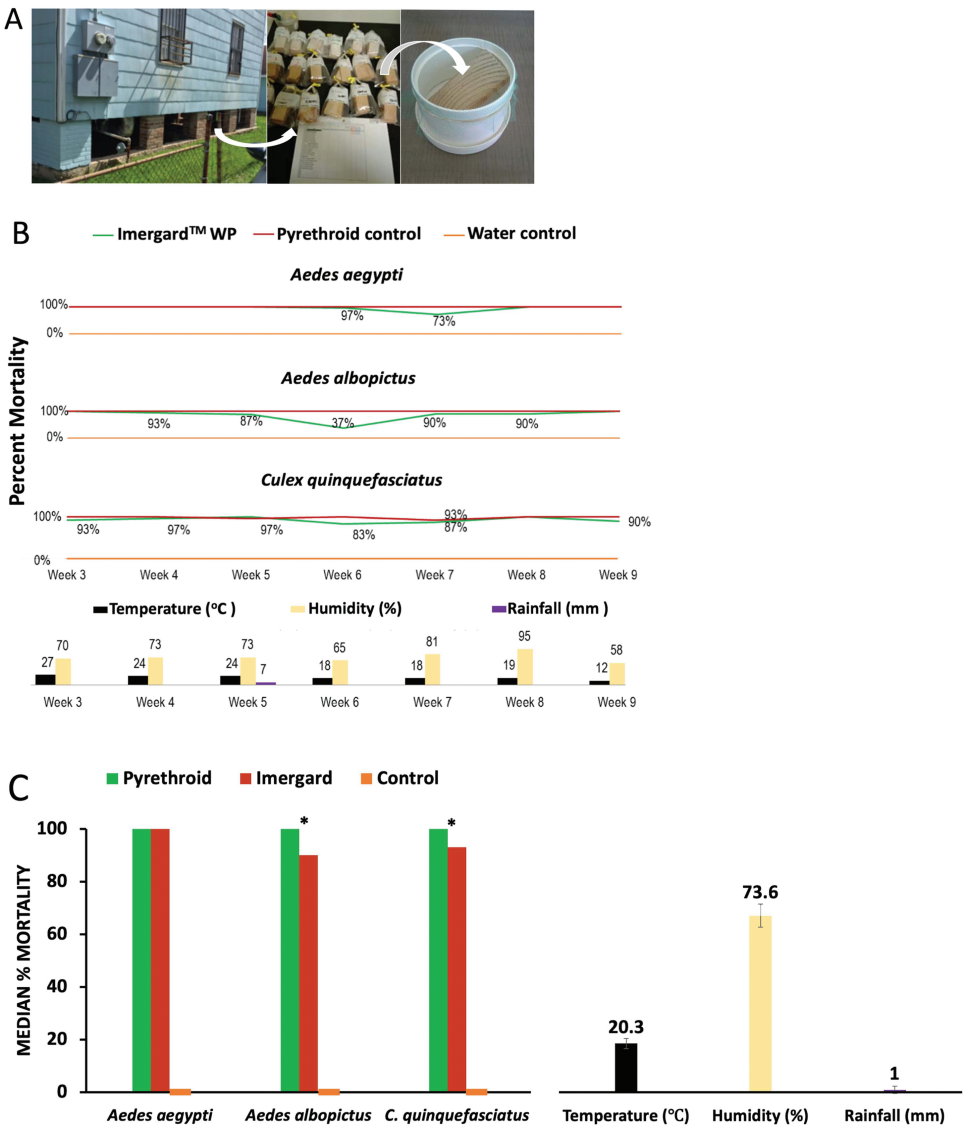


Fig. 4. Efficacy of Imergard™ WP, the pyrethroid lambda-cyhalothrin, and the water control on the mortality of *Aedes aegypti*, *Aedes albopictus*, and *Culex quinquefasciatus* applied to wood chips and then placed under stilt houses in New Orleans, Louisiana. (A: left to right) stilt house, treated wood chips in collection bags, and the architecture for the mosquito mortality assay, (B) weeks 3–9 mortality data for the three mosquito species and climatic conditions, and (C) bar graph on left showing median percent mortality for each species, presence of asterisk indicates significant difference between Imergard and pyrethroid ($P < 0.05$, Mann–Whitney *U*-test), no mortality was observed for the water control; bar graph on the right showing the average temperature, humidity, and rainfall during weeks 3–9.

of the body. SEMS of the negative controls had no extraneous material on the mosquito. We do not understand yet the exact amount of ImG needed on the mosquito and where it must be transferred for death. However, considering the size of the mosquitoes and the levels seen by SEM on the insect, only small amounts cause death. At the rate of our applications, there is likely enough material in theory in a single application for long-term control. We do not have data on the minimal concentration needed on a substrate to cause death. We would expect like for any insecticide, the composition of the treated surface and the method of application will have an impact on efficacy. However, based on our laboratory studies where the MI was applied in ethanol on glass versus by spray application in water on wood and where in both cases, we obtained high-percentage mortality, Imergard™WP is likely to be efficacious by a variety of application methods on a variety of different substrates.

Additional studies are needed to better understand the mechanism of action of Imergard WP on mosquitoes. Most likely, the transfer to the body is the result of an electrostatic differential between the treated surface and the insect and by direct contact of the tarsi with the MI-treated substrate. It is surprising that so little of the material is found on most of the body; it should not be concluded, however, that high-density levels are indicative of the major site of action of the MI_{VR} on the insect. There was no indication in the SEM analysis of physical disruption of the cuticle surface. It is clear that abrasion is not a factor in causing death since the mosquitoes once they land on the surface are not active and grooming. The obvious similarities of Imergard WP to diatomaceous earth both acting as dusts with a slow rate of kill suggest that the mechanism of Imergard WP is similar to diatomaceous earth, dehydration. More research is needed to confirm this. However, in studies with houseflies, the time to death with Imergard WP is shorter when humidity decreases (Chen et al., personal communication).

Imergard WP will provide a new, mechanical mode of action for mosquito control different from the current chemical insecticides

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used for vector management. The modes of action of current chemical insecticides used in Africa as residual wall sprays have a systemic action on the insect (WHO 2020b). In this respect, Imergard WP could be an important tool for mosquito resistance management. Imergard WP occurs naturally; there is essentially a limitless supply (=VR), is relatively safe (mammalian toxicity of Perlite is >10 g/kg, Maxim et al. 2014, and used in toothpaste), can be applied in water with typical spray equipment used in vector control, is heat stable (unlike chemistry), should have an indefinite shelf life and stability once applied (as long as not washed off), and is suitable as an indoor and outdoor residual spray for mosquito control. Additional work is needed to determine its activity against resistant mosquitoes and other vector arthropods like filth flies, sand flies, ticks, chiggers, etc. It is expected that mosquito resistance to chemical insecticides will not impact Imergard WP because of its mechanical mode of action. Additional research is needed to examine the use of this technology in Africa as a residual wall spray, its potential community level control of mosquitoes and reduction of malaria infections, duration of control compared with chemistry under field conditions, and its mode of action.

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(54) **Title:** TRAPPING BED NET FOR MOSQUITO CONTROL

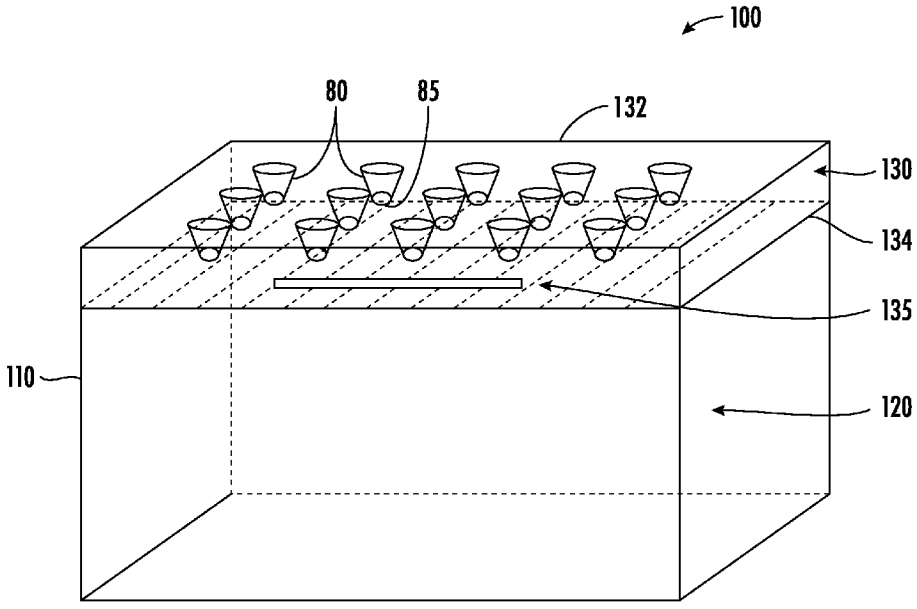


FIG. 1

(57) **Abstract:** A mosquito control net includes an occupant chamber and a trap chamber disposed over the occupant chamber. The trap chamber has one or more openings disposed on an outer surface thereof, through which mosquitos enter the trap chamber, the openings having receptacles attached therein and extending within the trap chamber to inhibit egression of mosquitos from the trap chamber after entry. A method for preventing mosquito bites for a subject can include providing such a mosquito control net and having the subject occupy it. A kit of parts for converting a conventional mosquito control net into a trapping mosquito control net can comprise a funnel-shaped receptacle surrounded by a bag, the bag being substantially impermeable to mosquitos and the receptacle and/or the bag being attachable to a top surface of the conventional mosquito control net to form a trap compartment.

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DESCRIPTION

TRAPPING BED NET FOR MOSQUITO CONTROL

CROSS-REFERENCE TO RELATED APPLICATION

This application claims benefit of and priority to U.S. Provisional Patent Application Serial No. 62/883,625, which was filed on August 6, 2019, the disclosure of which is incorporated by reference herein in its entirety.

TECHNICAL FIELD

The presently disclosed subject matter relates to apparatuses and methods for preventing the spread of mosquito-transmitted diseases.

DETAILED DESCRIPTION

Malaria transmitted by mosquitos is the leading cause of morbidity and mortality for humans on a global basis, especially in sub-Saharan Africa, with 212 million cases estimated by the World Health Organization (WHO) and about 429,000 deaths each year from this disease. One major stride in malaria prevention has been the use of long-lasting insecticide treated nets (LLINs). Mosquitos land on the nets at night, a small amount of the insecticide transfers to the mosquito, and they die. About 50 million nets are sold each year in Africa and an estimated 663 million cases of malaria have been averted in sub-Saharan Africa since 2001 due to the increase in deployments of malaria control interventions. However, the successes gained with long-lasting pyrethroid treated bed-nets is now in jeopardy because of wide-spread, pyrethroid-resistance in mosquitos, and they are no longer effective in killing mosquitos. Malaria parasites have a complex life cycle, which presents a significant obstacle in the development of an effective vaccine. Developing a single new chemistry for these nets would cost 100 million dollars or more with no assurance of success or prevention of future resistance to the new insecticide.

Mosquitos that are resistant to insecticides, however, are not resistant to (e.g., remain susceptible to) trapping. Thus, there is an urgent need for developing less expensive devices and methods to avoid mosquito bites that do not require the use of chemical insecticides. The presently disclosed

subject matter addresses these and other obstacles to preventing the transmission of many commonly mosquito-borne diseases.

SUMMARY

Accordingly, it is an object of the presently disclosed subject matter to provide apparatuses and methods for trapping and killing mosquitos. This and other objects are achieved in whole or in part by the presently disclosed subject matter.

An insecticide-free trapping net with two compartments is provided herein, this net including a lower sleeping, or occupant, chamber and an upper mosquito trap chamber containing cone-like funnels as mosquito entry points into the trap chamber. 3-D knitting may be used for cone construction.

The subject matter disclosed herein prevents mosquitos from biting people while they sleep, thus reducing the spread of mosquito-borne illnesses. The disclosed subject matter also kills mosquitos without chemicals with a high efficiency rate compared to conventional mosquito nets with insecticides added to the net by any method.

A trapping bed net, referred to as a "T-Net", is disclosed herein. The T-Net is used to control mosquitos by collecting them in a trap chamber each night, where they die in a few hours from desiccation and or starvation. Because there is no new chemistry to develop, the route to market is rapid and the cost for product development minimal. An aspect of the device is a cone, particularly cone (a fabric or knitted cone, plastic cone, paper cone, or any other material-made cone) which enhances trap efficacy and prevents mosquitos from escaping the trap. The presently disclosed subject matter also includes the use of any fabric type irrespective of the method used to make the fabric, for example a weave versus a knit, any other material used, for example plastic, paper, carbon fiber, etc., and any shape in addition to the shape of a cone, where the shape increases the probability of the mosquito entering into the trap chamber but reduces the probability of the mosquito leaving the trap chamber once in the trap. The trap chamber is defined as any space of textile construction but is not limited and could be made of other materials like plastics, paper, etc. that, once the mosquitos are in the trap, they cannot exit the trap and will die from starvation.

It is to be further understood that although the net disclosed herein is referred to as a "bed net", the mosquito trapping net can be used in a variety of applications, such as eating, working, playing, etc., and the term "bed" should not be construed as limiting.

An object of the presently disclosed subject matter having been stated above, other objects and advantages of the presently disclosed subject matter will become apparent to those of ordinary skill in the art after a study of the following description of the presently disclosed subject matter and non-limiting figures, which form part of the instant disclosure.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a schematic isometric view of an example embodiment of a net for trapping mosquitos to prevent an occupant within the net from being bitten by one or more mosquitos.

FIG. 2A is a schematic top view of another example embodiment of a net for trapping mosquitos to prevent an occupant within the net from being bitten by one or more mosquitos.

FIG. 2B is a schematic side view of the example embodiment shown in FIG. 2A.

FIG. 3A is a schematic top view of another example embodiment of a net for trapping mosquitos to prevent an occupant within the net from being bitten by one or more mosquitos.

FIG. 3B is a schematic side view of the example embodiment shown in FIG. 3A.

FIG. 4A is a schematic top view of another example embodiment of a net for trapping mosquitos to prevent an occupant within the net from being bitten by one or more mosquitos.

FIG. 4B is a schematic side view of the example embodiment shown in FIG. 4A.

FIG. 5 is an isolated perspective view of an example embodiment of a woven or mesh conical structure suitable for use in any of the example embodiments of the nets disclosed in FIGS. 1-4B.

FIG. 6A is a schematic perspective view of a net for trapping mosquitos therein according to any of the embodiments disclosed herein positioned within an example sleeping unit.

FIG. 6B is a schematic illustration of an example flight path of a mosquito entering the sleeping unit of FIG. 6A.

FIG. 7 is a schematic illustration showing mosquitos entering a frustoconically-shaped region.

FIG. 8A is a plot of the number of mosquitos captured as a function of the number of mosquitos present for the example embodiments of the nets shown in FIGS. 2A-4B.

FIG. 8B is a plot of the number of mosquitos captured as a function of the surrounding volume throughout which the mosquitos are dispersed for the example embodiments of the nets shown in FIGS. 2A-4B.

FIG. 9 is a table of data for a control mosquito net and the example embodiments of the nets shown in FIGS. 2A-4B.

FIG. 10A is a plot of the mean entry rate of mosquitos into the example sleeping unit of FIG. 6A, in which one of the nets shown in FIGS. 2A-4B, or a control net, were positioned with a human being present within each net.

FIG. 10B is a plot of the mean exit rate of mosquitos from the example sleeping unit of FIG. 6A, in which one of the nets shown in FIGS. 2A-4B, or a control net, were positioned with a human being present within each net.

FIG. 10C is a plot of the mean predicted mortality rate of mosquitos for each of the example embodiments of the nets shown in FIGS. 2A-4B, which were each installed within an example sleeping unit of FIG. 6A and had a human present within each net.

FIG. 10D is a plot of the mean actual mortality rate of mosquitos for each of the example embodiments of a control net and the nets shown in FIGS. 2A-4B, which were installed each within an example sleeping unit of FIG. 6A and had a human present within each net.

FIG. 11A is a schematic side view of a sleeping unit of FIG. 6A, in which a conventional mosquito control net known from the prior art is installed.

FIG. 11B is a schematic side view of a sleeping unit of FIG. 6A, in which a mosquito control net according to one of the example embodiments shown in FIGS. 1-4B is installed.

FIG. 12 is a plot showing the increase in mosquito mortality for the mosquito control nets shown in FIGS. 2A-4B, plotted as a multiple of the increase over the mosquito control provided by a conventional mosquito control net known from the prior art.

FIG. 13A is an exploded view of an example embodiment of a kit of parts for converting a conventional mosquito control net into a trapping mosquito control net.

FIG. 13B is an assembled view of the kit of parts of FIG. 13A.

FIG. 14 is a schematic side view of an example embodiment of a trapping mosquito control net with the kit of parts of FIGS. 13A and 13B attached thereto, the net being positioned over a subject contained within an interior thereof.

FIG. 15A is a top view of an example embodiment of a trapping mosquito control net with a plurality of kits of parts of FIGS. 13A and 13B attached thereto.

FIG. 15B is a schematic side view showing the arrangement of the various parts of the kit of parts of FIGS. 13A and 13B as the kit of parts is installed on a conventional mosquito control net to convert it into a trapping mosquito control net.

FIG. 16 is a schematic side view of mosquitos entering and being trapped within the trap chamber formed by the kit of parts of FIGS. 13A and 13B, which is attached to a converted trapping mosquito control net with a subject contained within an interior thereof to lure mosquitos into the trap chamber.

DETAILED DESCRIPTION

Mosquitos are attracted to carbon dioxide and other human odors. These odorants emitted from the body are warmer than ambient air and rise from its source. About 75% of mosquitoes follow this odorant plume and are attracted to the top surface of a net in use (e.g., installed in position over a sleep surface, such as a bed). As such, an insecticide-free trapping net with two compartments is provided herein, this net including a lower sleeping, or occupant, chamber and an upper mosquito trap chamber containing cone-like funnels as mosquito entry points into the trap chamber. 3-D knitting may be

used for cone construction. Proof of concept was investigated in the laboratory and then under field conditions with wild-type, resistant *An. gambiae* mosquitoes in Tiassale, Côte d'Ivoire (Africa), data of which is shown in FIGS. 8A-12.

The presently disclosed subject matter will now be described more fully hereinafter with reference to the accompanying figures, in which representative example embodiments are shown. The presently disclosed subject matter can, however, be embodied in different forms and should not be construed as being limited to the embodiments set forth herein. Rather, these embodiments are provided as examples so that this disclosure will be thorough and complete, and will fully convey the scope of the embodiments to those skilled in the art. Certain components in the Figures and Examples are not necessarily to scale, emphasis instead being placed upon illustrating the principles of the presently disclosed subject matter (in some cases schematically).

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this presently described subject matter belongs. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety.

While the following terms are believed to be well understood by one of ordinary skill in the art, the following definitions are set forth to facilitate explanation of the presently claimed subject matter.

Following long-standing patent law convention, the terms "a", "an", and "the" refer to "one or more" when used herein, including in the claims.

As used herein, the term "about", when referring to a value or an amount, for example, relative to another measure, is meant to encompass variations of in some embodiments ±20%, in some embodiments ±10%, in some embodiments ±5%, in some embodiments ±1%, and in some embodiments ±0.1% from the specified value or amount, as such variations are appropriate. The term "about" can be applied to all values set forth herein.

As used herein, the term "and/or" when used in the context of a listing of entities, refers to the entities being present singly or in combination. Thus, for example, the phrase "A, B, C, and/or D" includes A, B, C, and D

individually, but also includes any and all combinations and sub-combinations of A, B, C, and D.

The term "comprising", which is synonymous with "including," "containing," or "characterized by" is inclusive or open-ended and does not exclude additional, unrecited elements or method steps. "Comprising" is a term of art used in claim language which means that the named elements are present, but other elements can be added and still form a construct or method within the scope of the claim.

As used herein, the phrase "consisting of" excludes any element, step, or ingredient not specified in the claim. When the phrase "consists of" appears in a clause of the body of a claim, rather than immediately following the preamble, it limits only the element set forth in that clause; other elements are not excluded from the claim as a whole.

As used herein, the phrase "consisting essentially of" limits the scope of a claim to the specified materials or steps, plus those that do not materially affect the basic and novel characteristic(s) of the claimed subject matter.

With respect to the terms "comprising", "consisting of", and "consisting essentially of", where one of these three terms is used herein, the presently disclosed and claimed subject matter can include the use of either of the other two terms.

As used herein, "significance" or "significant" relates to a statistical analysis of the probability that there is a non-random association between two or more entities. To determine whether or not a relationship is "significant" or has "significance", statistical manipulations of the data can be performed to calculate a probability, expressed in some embodiments as a "p-value". Those p-values that fall below a user-defined cutoff point are regarded as significant. In some embodiments, a p-value less than or equal to 0.05, in some embodiments less than 0.01, in some embodiments less than 0.005, and in some embodiments less than 0.001, are regarded as significant.

In some embodiments, the subject treated according to the presently disclosed subject matter is a human subject, although it is to be understood that the methods described herein are effective with respect to all mammals. More particularly, provided herein is the treatment of mammals, such as humans, as well as those mammals of importance due to being endangered

(e.g., Siberian tigers), of economic importance (animals raised on farms for consumption or another use, such as the production of wool, by humans) and/or social importance (animals kept as pets or in zoos) to humans, for instance, carnivores other than humans (e.g., cats and dogs), swine (e.g., pigs, hogs, and wild boars), ruminants (e.g., cattle, oxen, sheep, giraffes, deer, goats, bison, and camels), and horses. Thus, embodiments of the systems and methods described herein include the treatment of livestock and pets.

A first example embodiment of a mosquito control net, generally designated **100**, is shown in FIG. 1. The net **100** has an occupant chamber, generally designated **120**, and a trap chamber, generally designated **130**. The trap chamber **130** has one or more openings in an outer (e.g., top, or upper) surface **132** of the trap chamber **130** and/or the net **100**. The trap chamber **130** is separated from the occupant chamber **120** by a barrier layer **134**, which is made from a textile material that mosquitos cannot pass through, such that mosquitos within the trap chamber **130** cannot pass into the occupant chamber **120** through the barrier layer **134**. In this embodiment, the net **100** comprises an outer shell **110** forming an exterior of both the trap chamber **130** and the occupant chamber **120**. As such, the trap chamber **130** is integrated, along with the occupant chamber **120**, into the outer shell **110**. As such, the barrier layer **134** extends in the form of a substantially continuous and uninterrupted layer between the lateral sides and/or surfaces of the outer shell **110** to define the occupant chamber **120** and the trap chamber **130**. In some embodiments, part or all of the textile material used to construct at least the portion of the outer shell **110** defining the occupant chamber **120** and/or the barrier layer **134** can be made of a three-dimensional "spacer fabric" (e.g., a fabric having two layers separated by an inner spacing material, in the manner of a sandwich construction.)

The openings are formed in the outer surface **132** of the trap chamber **130**, which entirely covers the barrier layer **134**, which can also be referred to as an inner layer of the occupant chamber **120**. In some embodiments, the outer surface **132** of the trap chamber **130** can only partially cover the barrier layer **134**. In some embodiments, the trap chamber **130** can be disposed partially or entirely over one or more sides of the occupant chamber **120**, for example, such that all or some of the outer walls of the outer shell **110**

comprise at least a portion of one or more trap chambers **130**. In some such embodiments, the barrier layer **134** can extend in both the vertical and/or horizontal directions, as necessary, to create a barrier that is impermeable to mosquitos between the trap chamber **130** and the occupant chamber **120**.

The openings formed in the outer surface **132** of the trap chamber **130** can take a number of forms, such as, for example, holes formed or cut into the textile material that forms the outer surface **132**. In some embodiments, the openings formed in the outer surface **132** are a textile material that is more coarsely woven (e.g., has larger voids in the weave pattern) than the textile material that forms the outer shell **110**, the textile material that is more coarsely woven being sewn into the textile material that is more finely woven to allow mosquitos to pass through the textile material that is more coarsely woven. In some embodiments, the textile material of at least the outer surface **134** is woven such that voids in the weave pattern are large enough for a mosquito to pass through in a first direction (e.g., towards the interior of the trap chamber **130**), but small enough to prevent the mosquito passing therethrough in a second direction, opposite the first direction (e.g., away from the interior of the trap chamber **130**). At least the portions of the outer shell **110** defining the occupant chamber **120** are made from a breathable textile material (e.g., a woven fabric with holes that are too small for mosquitos to pass through).

In the example embodiment shown in FIG. 1, the openings are three-dimensional receptacles **80** having a generally conical shape, however, in some embodiments, the receptacles **80** can have square, rectangular, hemispherical, triangular, or any other suitable shape(s). An example and non-limiting embodiment of a receptacle **80** that tapers towards the mouth **85** of the receptacle **80** is shown in FIG. 5 and is in the shape of, and acts similarly to, a funnel and can be made from any suitable material. In the embodiment shown in FIG. 5, the receptacle **80** is made of a woven material. Receptacles **80** each comprise a mouth **85**. The net **100** and/or portions thereof, such as, for example, the trap chamber **130** can be made from fiber or yarn used in conventional nets and/or any other suitable material, fiber or yarn. Materials can include, for example, woven, non-woven, braid, netting, and lace fabrics,

as well as "formed" (i.e., monolithic) material, such as, but not limited to, a plastic material.

A second example embodiment of a mosquito control net, generally designated **200**, is shown in FIGS. 2A and 2B. The net **200** has an occupant chamber, generally designated **220**, a trap chamber, generally designated **230**, and walls **236**. The trap chamber **230** has one or more (e.g., a plurality of) openings in an outer (e.g., top, or upper) surface **232** of the trap chamber **230** and/or the net **200**. The trap chamber **230** is separated from the occupant chamber **220** by a barrier layer **234**, which is made from a textile material that mosquitos cannot pass through, such that mosquitos within the trap chamber **230** cannot pass into the occupant chamber **220** through the barrier layer **234**. In this embodiment, the net **200** comprises an outer shell **210** forming an exterior of both the trap chamber **230** and the occupant chamber **220**. As such, the trap chamber **230** is integrated, along with the occupant chamber **220**, into the outer shell **210**. As such, the barrier layer **234** extends in the form of a substantially continuous and uninterrupted layer between the lateral sides and/or surfaces of the outer shell **210** to define the occupant chamber **220** and the trap chamber **230**. In some embodiments, part or all of the textile material used to construct at least the portion of the outer shell **210** defining the occupant chamber **220** and/or the barrier layer **234** can be made of a three-dimensional "spacer fabric" (e.g., a fabric having two layers separated by an inner spacing material, in the manner of a sandwich construction.)

The openings are formed in the outer surface **232** of the trap chamber **230**, which entirely covers the barrier layer **234**, which can also be referred to as an inner layer of the occupant chamber **220**. The openings in the outer surface **232** are grouped in a region of the net **200** corresponding to the position at which a user's head is positioned while sleeping within the net **200**. The attraction of mosquitos through the receptacles **80** into the trap chamber **230** is a result of attraction of the mosquitos to carbon dioxide and other odors from human respiration. As such, in this example embodiment, receptacles **80** were only positioned on the head end of the net **200** with a circular aggregate of 7 frustoconically-shaped receptacles **80** spaced about 30 cm from the end of the net **200.** By way of example and not limitation, each receptacle has a 10 cm diameter inlet, an 8 cm depth, and a 1.8 cm diameter exit, or outlet, as

measured at the cross-section of the mouth **85**. The receptacles **80** were attached around the midline of the long axis of the outer surface **232** of the trap compartment **230**, which has an exemplary, but non-limiting, depth of 25 cm.

A third example embodiment of a mosquito control net, generally designated **300**, is shown in FIGS. 3A and 3B. The net **300** has an occupant chamber, generally designated **320**, a trap chamber, generally designated **330**, and an upper wall **322**. In this embodiment, the net **300** comprises an outer shell **310** forming an exterior of both the trap chamber **330** and the occupant chamber **320**. As such, the trap chamber **330** is integrated, along with the occupant chamber **320**, into the outer shell **310**. As such, the barrier layer **334** extends in the form of a substantially continuous and uninterrupted layer between the lateral sides and/or surfaces of the outer shell **310** to define the occupant chamber **320** and the trap chamber **330**. In some embodiments, part or all of the textile material used to construct at least the portion of the outer shell **310** defining the occupant chamber **320** and/or the barrier layer **334** can be made of a three-dimensional "spacer fabric" (e.g., a fabric having two layers separated by an inner spacing material, in the manner of a sandwich construction.)

The net **300** has openings formed in the outer surface **332** of the trap chamber **330**, which entirely covers the barrier layer **334**, which can also be referred to as an inner layer of the occupant chamber **320**. To investigate increasing the trapping rate and add additional functionality, the net **300** has only a single large conically-shaped receptacle **80**, which, by way of example and not limitation, has a 15 cm diameter inlet, a depth of 12 cm, and a 3 cm diameter exit, or outlet, as measured at the cross-section of the mouth **85**. As shown in FIGS. 3A and 3B, the receptacle **80** is attached to the outer surface **332** to extend within a trap chamber **330**, which is in the form of a cylindrically-shaped bag have an exemplary, but non-limiting, depth of 25 cm, as measured from the outer surface **332** to the bottom surface **334**, and an exemplary, but non-limiting, diameter of 25 cm, as measured between opposing sides of the wall **336**. By way of example and not limitation, a trap chamber **330** is attached on the outer surface **332** of the net 30 cm from the longitudinal edge of the net **300**. The construction and configuration of the net **300** is advantageous in

reducing material costs for construction and allowing for the head space between the sleeper and outer surface **332** of the net **300**, so as to effectively increase the odorant concentration on the top of the net, as well as adding practical functionality to the bed net design. The design of the net **300** could advantageously be used to retrofit any conventional net, including those already deployed in homes.

A fourth example embodiment of a mosquito control net, generally designated **400**, is shown in FIGS. 4A and 4B. The net **400** has an occupant chamber, generally designated **420**, a trap chamber, generally designated **430**, and walls **436**. The trap chamber **430** has one or more (e.g., a plurality of) openings in an outer (e.g., top, or upper) surface **432** of the trap chamber **430** and/or the net **400**. The trap chamber **430** is separated from the occupant chamber **420** by a barrier layer **434**, which is made from a textile material that mosquitos cannot pass through, such that mosquitos within the trap chamber **430** cannot pass into the occupant chamber **420** through the barrier layer **434**. In this embodiment, the net **400** comprises an outer shell **410** forming an exterior of both the trap chamber **430** and the occupant chamber **420**. As such, the trap chamber **430** is integrated, along with the occupant chamber **420**, into the outer shell **410**. As such, the barrier layer **434** extends in the form of a substantially continuous and uninterrupted layer between the lateral sides and/or surfaces of the outer shell **410** to define the occupant chamber **420** and the trap chamber **430**. In some embodiments, part or all of the textile material used to construct at least the portion of the outer shell **410** defining the occupant chamber **420** and/or the barrier layer **434** can be made of a three-dimensional "spacer fabric" (e.g., a fabric having two layers separated by an inner spacing material, in the manner of a sandwich construction.)

The openings are formed in the outer surface **432** of the trap chamber **430**, which entirely covers the barrier layer **434**, which can also be referred to as an inner layer of the occupant chamber **420**. To investigate the relationship between catch rate and the rate of egression from the trap chamber **430**, the net **400** has a plurality of (e.g., four) conically-shaped receptacles **80**, each of which has an exemplary, but non-limiting, 15 cm diameter inlet, an exemplary, but non-limiting, depth of 12 cm, and an exemplary, but non-limiting, 3 cm diameter exit, or outlet, as measured at the cross-section of the mouth **85**. As

shown in FIGS. 4A and 4B, each of the receptacles **80** is attached to the outer surface **432** to extend within the trap chamber **430**, which is generally in the form of a rectangular prism having an exemplary, but non-limiting, depth of 25 cm, as measured between the outer surface **432** and the barrier layer **434**. The receptacles are spaced substantially equidistantly and/or uniformly away from each other and the edges of the net **400**, such that the receptacles are spaced apart by a length L (e.g., 26 cm) and arranged lengthwise along the centerline of the net **400**.

The efficacy of the nets **200**, **300**, **400** was modeled to predict trapping rates, which are shown by the regression curves in FIGS. 8A and 8B. FIG. 8A shows a plot of the capture rate of the nets **200**, **300**, **400**, as a function of mosquitos entering a constant volume in which the nets **200**, **300**, **400** are installed with a human positioned within the nets **200**, **300**, **400**. It can be seen that the net **400** has the best capture rate, followed by net **200**, with net **300** having the lowest capture rate. FIG. 8B shows a plot of the capture rate of the nets **200**, **300**, **400**, as a function of a constant number of mosquitos being present within a space having a variable volume, in which the nets **200**, **300**, **400** are installed with a human positioned within the nets **200**, **300**, **400**. It can be seen that, while the capture rate for all of the nets increases drastically as a function of the volume of the area in which the nets **200**, **300**, **400** are installed, the net **300** has the best capture rate, followed by net **400**, with net **200** having the lowest capture rate.

In order to validate the results of the nets **200**, **300**, **400**, field trials were conducted, in which the efficacy of the nets **200**, **300**, **400** was compared against that of a conventional net, a Permanent 2.0 LLIN, which is the most used LLIN in Africa. These field trials were conducted in WHO-approved experimental huts (see, e.g., FIG. 6A) in the locality of Tiassale, wherein wild *An. Gambiae* malaria-bearing mosquitos are insecticide-resistant, with human subjects sleeping under the control net and the nets **200**, **300**, **400** each night. The estimated flight of a mosquito within such a hut is shown in FIG. 6B. The data obtained during these field trials is shown in FIG. 9.

The mean entry number was recorded and is shown in FIG. 10A. A total of 53, 139, 175 and 156 *An. gambiae sl* mosquitoes were collected over 14 nights (representing 14 observations = 14 replicates), respectively, for the

control, net **200**, net **300**, and net **400**. The mean number of mosquitos entering each hut per night was 3.8 (σ= 2.96) for the control net, the lowest entry rate in the field trial, followed by 9.9 (σ= 10.1) for the net **200**, 12.5 (σ= 10.5) for the net **300**, and 11.1 (σ= 8.3) for the net **400**. The differences were not statistically significant between the nets **200**, **300**, **400**, but a significant difference was observed between the control net and each of the nets **200**, **300**, **400** ($P<0.05$).

The mean exit rate was recorded and is shown in FIG. 10B. The control net not only showed the lowest entry rate but also the highest mean exit rate of mosquitos per night, 77.2% (σ = 29.4) and was statistically significantly different from that of the nets **200**, **300**, **400**. In nets **200**, **300**, a mean mosquito exit rate per night of 47.3% (σ= 27.7) and 45.7% (σ= 30.6), respectively, were recorded. In net **400**, a mean mosquito exit rate of 26.7% (σ= 20.7) was recorded, which was the lowest mean exit rate. No statistically significant differences in exit rates was observed between the three nets **200**, **300**, **400**.

The mean mortality rate was also recorded and is shown in FIG. 10D. The lowest mortality rate was recorded with the control net, 14.3% (σ= 17.9). The mean mortality rate of the control net was statistically significantly lower than for all of the nets **200**, **300**, **400**. The mortality rates for the nets **200**, **300** were 39.1% (σ = 30.5) and 39.8% (σ = 26.0), respectively, with no statistical significant differences between these two nets ($P> 0.05$). The highest mean mosquito mortality rate per hut per night was for the net **400** at 61.9% (σ= 22.1). This difference was higher than that of the nets **200**, **300**, and the control net ($P <0.05$).

The mean mortality rate for the nets **200**, **300**, **400** were predicted and are shown in FIG. 10C. It was predicted that the trap number for nets **200**, **300**, **400**, respectively, at 2.87, 1.41 and 5.02 corresponding to trapping rates of 28.9%, 11.3% and 45.06%. The relative trap efficiency between nets **200**, **300**, **400** predicted was in agreement but underestimated the actual trap efficacy in the field trials, shown in FIG. 10D. This was expected since the predicted data did not take into consideration the attractants from the sleeper within the occupant chamber of the net. For the net **200**, there was a much greater trap rate than predicted, suggesting that receptacle positioning above

the sleeper head likely is responsible for a greater number of trapped mosquitoes than the other receptacles in the net **400**. At the same time, the higher efficacy of the net **400** where the receptacles run along the long top axis of the net **400** confirms that odorants from other parts of the body increase trap efficacy.

The nets **200**, **300** had a 2.7-times (sometimes referred to as a "fold" increase) greater kill rate than the control net, while the net **400** had a 4.3-times greater kill rate than the control net. However, there is more to consider in comparing these different bed net technologies and the impact on mosquito mortality in the community for the nets **200**, **300**, **400** is actually much greater than shown by these data. The deterrence rate was much lower and the repellency rate much higher for the control net compared to any of the nets **200**, **300**, **400**. FIG. 11A is a schematic side view of a conventional mosquito control net **10**, which has an insecticide applied thereto and is installed within an example residential unit, generally designated **500**. As shown in FIG. 11A, if the mosquitos do not enter the hut because of deterrence due to the presence of the insecticide on the control net and/or if the mosquitos enter the hut but are repelled before receiving a lethal insecticide dose from the control net, these mosquitos are not being controlled in the community at large, as they have not been neutralized and remain alive to circulate through the community.

FIG. 11B is a schematic side view of a net **100**, **200**, **300**, **400**, which is devoid of any insecticide and is installed within a substantially identical example residential unit **500** as is shown in FIG. 11A. As shown in FIG. 11B, mosquitos **50** enter the residential unit through an entryway, generally designated **510**, such as a window or doorway that is at least partially open, or ajar. Unlike in FIG. 11A, in which at least some proportion of mosquitos **50** are deterred from entering the residential unit **500** through the entryway **510** due to the presence of the insecticide on the conventional mosquito control net **10** and remain able to circulate in the community, in FIG. 11B there is no insecticide present and, as such, no deterrence to the entry of the mosquitos **50** through the entryway **510**, even for mosquitos **50** that are insecticide-resistant. As such, unlike in FIG. 11A, in FIG. 11B approximately all of the mosquitos who attempt to enter the residential unit **500** through the entryway

510 do actually enter the residential unit **500** and substantially no mosquitos are deterred from entering (e.g., by the presence of insecticide). As can be seen in FIG. 11B, live mosquitos are able to circulate within the residential unit **500**, external to the net **100**, **200**, **300**, **400**. Because of their inability to successfully feed from a host, some mosquitos (e.g., dead mosquitos **50D**) will die within the residential unit as a result of their inability to reliably locate the entryway **510** for egress from the residential unit **500**, even though these dead mosquitos **50D** were not trapped by the net **100**, **200**, **300**, **400**. Mosquitos **50** that are trapped and/or killed within the net **100**, **200**, **300**, **400** are generally not able to escape the net **100**, **200**, **300**, **400**, much less the residential unit **500**, to continue circulating throughout the community at large.

In determining a theoretical model to predict the efficacy of the nets **100**, **200**, **300**, **400** disclosed herein, it was assumed that the flight track of mosquitoes is random, the diameters of the receptacles **80** into the trap chambers **130**, **230**, **330**, **430** are large enough not to affect the flight path of mosquitos, and that the trapping of the mosquitos does not perturb the flight velocity of any remaining untrapped mosquitoes present in the residential unit **500**.

FIG. 6A shows an example embodiment of a residential unit, generally designated **500**, with one or more entryways **510** (e.g., windows and/or doors) formed in an exterior thereof for ventilation, and through which a mosquito would be able to pass if not closed. As shown in FIG. 6A, a net **100**, **200**, **300**, **400** according to one of the example embodiments disclosed herein is installed within the residential unit **500**. FIG. 6B schematically shows the flight path **52** of a mosquito **50** after entering a residential unit **500** through, for example, and entryway **510**, as having both random and targeted aspects, as the mosquito **50** is generally able to hone in on the top surface of the net **100**, **200**, **300**, **400** based on the presence of a CO_2 plume emanating therefrom.

FIG. 7 schematically shows an assumed hole area dA, a mosquito **50** that is at a distance vdt from the hole and is moving at a speed v at an angle θ from the normal surface toward the area dA. All mosquitos **50** within a parallelepiped volume (e.g., within receptacle **80**) around the area dA moving toward the hole with speed v will pass through the mouth of the cone in the time interval dt. Therefore, the total number of mosquitos **50** that will pass

through the area dA in the time interval dt can be expressed according to the equation:

$$N_A = \rho v cos\theta dA dt \quad (1)$$

where ρ is the mosquito density in the residential unit **500**.

Assuming the distribution of the speeds v of individual mosquitos **50** obeys the Maxwell-Boltzmann distribution, which was first defined and used for describing particle speeds in idealized gases, one integrated expression of the distribution relates particle density with average velocity. Similarly, the average trapped mosquito number per area per time is can then be expressed according to the equation:

$$\dot{N} = C\bar{v}\rho \quad (2)$$

where $\bar{v}$ is the average flight speed, ρ is the mosquito density in the residential unit **500** and C is a constant determined by the active behavior of the mosquito (i.e., 1/4 in ideal gas theory).

Since the majority of mosquitos in the residential unit will fly generally in the direction of the top (e.g., outer surface **132**) of the net, the work volume can be expressed according to the equation:

$$V_w = V - V_{net} - V_s \quad (3)$$

where V is the volume of the residential unit **500**, $V_{net}$is the volume of the net **100**, **200**, **300**, **400** and V_s is the volume surrounding the net **100**, **200**, **300**, **400**, except the volume on the top of the net **100**, **200**, **300**, **400**.

By assuming that mosquitos are distributed evenly on the top of the net **100**, **200**, **300**, **400**, the number of trapped mosquitos for each net **100**, **200**, **300**, **400** in the testing time t can be expressed according to the equation:

$$N = C \times a \times \frac{N_{ru} \times \bar{v}}{(V - V_{net} - V_S)} \times t \quad (4)$$

where N_{ru} is the total number of mosquitos in the residential unit **500**, C is a constant, and a is the bottom area of the cone.

Thus, the model for each of the nets **100**, **200**, **300**, **400** relates the number of mosquitos trapped with the container volume and flight velocity. If the number of mosquitos in a hut is variable and other parameters are held

constant, the predicted results of equation (**4**) are shown in FIG. 8A. if V_{hut} is treated as a variable, the predicted results of equation (4) is shown in FIG. 8B.

Overall relative mosquito control can be calculated according to the following equation:

$$Overall\ relative\ mosquito\ control\ = \frac{Mu}{(1 - \mathrm{D})\ x\ Mi}$$

where *Mu* is the mortality caused by the untreated net **200**, **300**, **400**, *e* is the mortality caused by the insecticide-treated control net; D is the deterrent effect (D=((*Eu*-*e*))/*Eu*); *Eu* is the mean entry number in the untreated net **200**, **300**, **400**; and *Ei* is the mean entry number in the insecticide-treated control net. The results of this equation are plotted in FIG. 12. The net **400** was determined to be 12.7x more efficacious than the control net, the net **300** was determined to be 9.1x more efficacious than the control net, and the net **200** was determined to be 7.1x more efficacious than the control net.

In some embodiments, the trapping portion of the bed net can be deployed as a single, larger funnel. Alternately, the trapping portion can be in the form of multiple smaller funnels. The receptacles **80** are disposed in the outer surface **132**, which is a top, or upper, surface of the net **100**, such that the receptacles **80** point downward (e.g., generally aligned with the direction of gravity). In some embodiments, each receptacle **80** can be surrounded by a separate trap chamber, such that the net **100** would have a plurality of trap chambers **100**, rather than one single trap chamber **130** extending across the outer surface **132** of the net **100**. In the illustrated embodiment of the net **100**, the receptacles **80** extend inwardly, into the trap chamber **130**, which is internal to the outer surface **132** to form a single large trap chamber **130** covering the top of the net **100**. In some embodiments, receptacles **80** can be made from a substantially rigid material to allow mosquitos to pass through the opening formed by the receptacles **80** and can be fixed on the lateral sides of the trap chamber **130**. A combination of any and/or all or portions of these embodiments is also provided.

In some embodiments, the net **100**, **200**, **300**, **400** does not use (e.g., is free, or devoid, of) chemical insecticides. The mosquitos become ensnared in the trap chamber **130**, **230**, **330**, **430** and are unable to escape, dying of desiccation.

In some embodiments, the net **100**, **200**, **300**, **400** is washable and foldable for flat storage and/or storage within a small package and is easily assembled and/or disassembled. The net **100**, **200**, **300**, **400** can include a sealable opening **135** (see FIG. 1) into the trap chamber **130**, **230**, **330**, **430** for emptying mosquito carcasses from the interior of the trap chamber **130**, **230**, **330**, **430**.

In some embodiments, the net **100**, **200**, **300**, **400** can be rapidly produced and deployed with minimal development and consumer cost. Studies show that the disclosed nets **100**, **200**, **300**, **400** are up to twelve (12) times or more efficacious than nets currently being used for mosquito control. In some embodiments, a trap chamber can be added to conventional nets that currently do not have a trap chamber, either by adding a trap chamber (e.g., with one or more suitably-shaped receptacles **80**) to the top surface (e.g., over the entire top surface, or a portion thereof) of the conventional net. In some embodiments, such conventional nets can be modified using receptacles **80** with individual trap chambers (see, e.g., **330**, FIGS. 3A, 3B) around each receptacle **80**, with one or more such individual trap chambers being mounted on and/or through the outer (e.g., top) surface of such a conventional mosquito control net.

In some aspects, the receptacles **80** of the net **100**, **200**, **300**, **400** comprise enhanced mosquito channeling geometry and/or improved netting construction. In other aspects, the nets **100**, **200**, **300**, **400** can be treated with mosquito attractants.

In further aspects, the nets **100**, **200**, **300**, **400** can be configured to optimize space in the occupant chamber about and/or around the head of the occupant of the net **100**, **200**, **300**, **400**. The net **100**, **200**, **300**, **400** can additionally be configured to optimize the shape and/or depth of the trap chamber **130**, **230**, **330**, **430** to minimize egression of the mosquitos. The receptacles **80** can be of any shape that promotes entry into the trap chamber **130**, **230**, **330**, **430** and reduces the probability of egression of the mosquitos from the trap chamber **130**, **230**, **330**, **430** after the mosquitos have passed therein through the mouth **85** of the receptacle **80**. In some embodiments, the receptacles **80** can be of different sizes, numbers, and/or positions on the net **100**, **200**, **300**, **400** to allow mosquitos to enter the trap chamber **130**, **230**,

330, **430**. The size and dimensions of the trap chamber **130**, **230**, **330**, **430** can be adjusted according to the measured results to maximize the number of mosquitos trapped. Also, the openings can be of different shapes, for example, round, square, rectangle, etc. In some embodiments, the receptacles **80** can comprise flaps to partially cover the inlet and/or outlet of the receptacles, or the receptacles **80** can be partially occluded by structures of different types, for example, by textile materials having a low density number of threads.

Mosquitos are highly attracted to carbon dioxide (CO_2). When a person is sleeping and relatively motionless, their exhalation plume of warm CO_2 rises, acting to attract mosquitos. Studies have shown that, when mosquito control nets are used over beds where people sleep, the majority of mosquito activity occurs at the top surface of the net. As such, the presently disclosed subject matter includes, in some embodiments, carbon dioxide channeling as part of the function of the nets **100**, **200**, **300**, **400** in increasing the efficacy thereof in trapping and killing mosquitos.

For openings, such as receptacles **80**, or other structures that promote mosquitos entering into the trap chamber , it has been determined that there is a preferred diameter of the opening and the number of openings relative to the diameter and also relative to the outside area of the trap chamber, the volume of the trap chamber, the shape of the trap chamber, and the presence of structures of different forms inside the trap chamber. In the example embodiments of the nets, **100**, **200**, **300**, **400** shown and discussed herein, the optimum diameter is defined with a possible variation around plus/minus 68%. These abovementioned parameters themselves, irrespective of the shape of the receptacle (e.g., a cone, or cone equivalent), affect both mosquito trapping and a low egression rate once the mosquitos have been trapped (e.g., have entered the trap chamber). This impacts the carbon dioxide plume and trapping efficacy and includes the carbon dioxide penetration rate of the textile material in which the trap chamber is constructed. Thus, the net **100**, **200**, **300**, **400** can be constructed in a manner to affect the path and/or presence of carbon dioxide. For example, in some embodiments, the sides (see, e.g., walls **236**, FIG. 2B) of the trap chamber can be constructed with a textile that does not readily transmit carbon dioxide.

Alternately, the sides and the top or just the top of the trap chamber can be constructed of a material that does not transmit carbon dioxide, forcing the carbon dioxide in the exhaled plume to pass through only the available openings and to advantageously increase the attraction rate of mosquitos entering the trap chamber. It is also possible to add additional structure into the interior of the trap chamber to both improve carbon dioxide channeling and at the same time reduce egression by providing barriers once the mosquito is in the trap.

According to the present disclosure, mosquitos seeking the source of the CO_2 plume enter a trap chamber on a top surface of the net, which has one or more openings in the outer surface. The openings are designed such that they are large enough for the mosquitos to easily pass through as they follow the plume of CO_2, but small enough that they cannot easily find the opening to exit. In a preferred embodiment, the openings are cones or funnels sewn into the outer fabric layer of the trap chamber. Mosquitos enter the funnel through an area that decreases in size as they pass through it. Once the mosquitos pass through the large diameter portion of the funnel, they travel through the mouth of the funnel toward the CO_2 and into the trap chamber. The mosquitos are not able to reach the subject in the occupant chamber, and they are generally prevented from leaving the trap chamber due to the small diameter of the mouth of the funnel on the interior of the trap chamber. The mosquitos then die of desiccation and/or starvation.

The net can be configured in different ways depending on a number of variables, such as, but not limited to, surroundings, mosquito species, and user preferences. For example, in the example embodiments of FIGS. 1-2B, 4A, and 4B, the size of the trap chamber can be designed to cover the entire top surface of the net or, as shown in the example embodiment of FIGS. 3A and 3B, the trap chamber can be formed in a more limited, smaller region of the net. In an alternate embodiment, the trap chamber can be produced as a standalone portion and then attached to a conventional net suitable to control mosquitos. Such a conventional net can either include or be free of (e.g., devoid of) chemical insecticide(s). In particular, the material from which the net is made can be configured to channel the carbon dioxide through the receptacles to help focus mosquito activity towards the entrances of the

receptacles. This direction of mosquito attraction can be achieved in some embodiments by making the top surface and/or sides of the trap compartment impermeable to carbon dioxide, as one example.

In some embodiments, various regions of the net(s) **100**, **200**, **300**, **400** can have the same or different color as other regions of the T-net. For example, since color influences the attraction of mosquitos, in some embodiments it may be advantageous to make the trap chamber **130**, **230**, **330**, **430** have a color that is darker than the color of another region (e.g., the occupant chamber **120**, **220**, **320**, **420**) to increase the attraction of mosquitos and/or to reduce the visibility of dead mosquitos.

Another advantage of the disclosed nets **100**, **200**, **300**, **400** is that each is easily washable with only water. Furthermore, mosquito carcasses can pass through the material of the nets **100**, **200**, **300**, **400** without damaging the net **100**, **200**, **300**, **400** itself. This allows easy maintenance and cleaning of such nets **100**, **200**, **300**, **400**. Since the nets **100**, **200**, **300**, **400** can be used without applying chemicals insecticides thereto, there is no danger of releasing pesticides into water systems while washing the mosquito carcasses out of the nets **100**, **200**, **300**, **400**. Another advantage is that the time and effort needed to wash the nets **100**, **200**, **300**, **400** to effectuate the removal of mosquito carcasses is reduced compared to conventional mosquito nets. For example, a two-minute rinse in a local stream has been shown to be effective in removing dead mosquito carcasses from the nets **100**, **200**, **300**, **400**.

Another advantage associated with the presently disclosed nets **100**, **200**, **300**, **400** is that it is thought that increasing the use of such physical barrier devices (and reducing the use of conventional nets having chemical insecticides applied thereto) could lead to an eventual decline in insecticide resistance in mosquitos, particularly in areas known to have chemically resistant mosquito populations. It is thought that this may allow the eventual reintroduction of currently known chemical insecticides into areas in which such insecticide-resistant mosquitos are prevalent.

Use of the nets **100**, **200**, **300**, **400** disclosed herein may also lead to increased use of nets for mosquito control in general. The usefulness of sleeping under a net is reinforced when the user empties the trap chamber

and observes the number of mosquitos trapped. This may encourage users to follow the usage directions properly and use such nets more frequently. Also, since the users are not exposed to a chemical insecticide, it is thought that this elimination in exposure to an insecticide might promote net use for users that want to avoid exposure to insecticide(s). If insecticides pose even a perceived health risk, whether known or not, the presently disclosed nets **100**, **200**, **300**, **400** eliminate this risk.

In some embodiments, at least a portion of the nets **100**, **200**, **300**, **400** is treated with an insecticide. In some such embodiments, only the sides (e.g., all of the sides) of the occupant chamber **120**, **220**, **320**, **420** are treated with one or more insecticides. In some embodiments, the insecticide is applied on the sides of the trap chamber **130**, **230**, **330**, **430** and the occupant chamber **120**, **220**, **320**, **420**, which can be advantageous because chemical-resistant mosquitos can be repelled by the insecticide on the sides of the net **100**, **200**, **300**, **400**. However, because even these insecticide-resistant mosquitos are still drawn towards and will follow the path of CO_2 plume, these insecticide-resistant mosquitos will nevertheless fly to the outer, or top, surface **132**, **232**, **332**, **432** of such nets **100**, **200**, **300**, **400**, into and through the receptacles **80**, and then into the trap chamber **130**, **230**, **330**, **430**, from which the mosquitos are unable to regularly find an exit, and in which the majority of the mosquitos that enter therein will die. Applying one or more insecticides to at least portions of the sides of the net as described hereinabove is further advantageous because, during typical use and over the useable life of such nets **100**, **200**, **300**, **400**, they will almost invariably develop holes from human use. As such, when the sides of the nets **100**, **200**, **300**, **400** are treated with insecticide(s), the insecticide-resistant mosquitos will still be repelled by the insecticide will not go through the damage-induced holes; Rather, these insecticide-resistant mosquitos will still fly into the trap compartment as described hereinabove. In such embodiments, mosquitos that are not insecticide-resistant will invariably come into contact with the side of such nets **100**, **200**, **300**, **400** and would be killed due to exposure to the insecticide. In some embodiments, the sides or bottom of the trap chamber **130**, **230**, **330**, **430** can be treated with insecticide(s) to kill mosquitos within the trap chamber **130**, **230**, **330**, **430** more quickly and/or to reduce the rate of success of

mosquitos trapped within the trap chamber **130**, **230**, **330**, **430** from finding a path out of the trap chamber **130**, **230**, **330**, **430**.

In some embodiments, the bed net is constructed by stamping the receptacles **80** (e.g., to be in the shape or a cone or a cone equivalent, including any receptacle shape that has the functions described herein) into the same or different type of material used for at least the outer, or top, surface **132**, **232**, **332**, **432** and/or of the outer shell **110**, **210**, **310**, **410** of the net **100**, **200**, **300**, **400**. This stamped material for the receptacles **80** can then be sewn into the net **100**, **200**, **300**, **400** or the stamping formed on the outer, or top, surface **132**, **232**, **332**, **432** of the trap chamber **130**, **230**, **330**, **430**. The material used for stamping the receptacles **80** can be any suitable material for forming the receptacle **80** and retaining the shape of the receptacle **80** over time and use (e.g., not deforming so that the mouth **85** of the receptacle(s) substantially retains its original cross-sectional shape). For example, some materials described hereinabove can be used for heat setting the receptacles in a desired shape (e.g., conical or frustoconical), or any other suitable yarn type used for heat setting. The presently disclosed subject matter is not limited to these particular materials but can also include others that impart these same characteristics both in immediate function and that are compatible to the function of the disclosed nets **100**, **200**, **300**, **400** (e.g., to trap and kill mosquitos). A heat press used for stamping can be configured to form single or multiple receptacles having a generally frustoconical shape and of any optimum size.

According to another example embodiment, a kit of parts for the net is also provided. In some embodiments, the kit of parts can comprise one or more of the presently disclosed nets **100**, **200**, **300**, **400** and a container for the one or more nets **100**, **200**, **300**, **400**. Each device in the kit can comprise a different configuration. In the example embodiments shown in FIGS. 13A-16, which will be described further herein, a kit of parts, generally designated **600**, is configured for converting a conventional (e.g., insecticide-laden) mosquito control net **10** into a trapping mosquito control net (e.g., one of nets **100**, **200**, **300**, **400**).

As can be seen in FIGS. 13A and 13B, the kit of parts **600** can include, for example and without limitation, a funnel-shaped receptacle **80** that is

surrounded by a trap chamber, generally designated **90**, which includes a bag **92** of any shape form the trap chamber **90** of the converted trapping mosquito control net using the kit of parts **600**. The kit of parts **600** can also include a collar, generally designated **70**. The collar **70** is generally annularly shaped and has threads **72** formed on an outer circumferential surface thereof, which are formed complementarily with internal threads **82** that are formed on an inner circumferential surface of an annularly shaped portion of the receptacle **80**, so that the threads **72** of the collar **70** can be twistably engaged with the internal threads **82** of the receptacle **80** to secure the collar **70** and the receptacle **80** together. The trap chamber **90** has an upper ring **94** that is made from a substantially rigid material (e.g., plastic, metal, and the like), to which the material that forms the bag **92** is rigidly (e.g., integrally) attached to prevent separation therefrom. The trap chamber **90** has threads **96** formed on and/or about an inner circumferential surface of the upper ring **94**. The threads **96** are shaped complementary to (e.g., to threadably engage and/or interlock with) threads **86** that are formed on an outer circumferential surface of the annular portion of the receptacle **80**, such that the entire trap chamber **90** may be threaded onto or off of the receptacle **80** without requiring the collar **70** and/or the receptacle **80** from being removed from the net **10**. This is advantageous in that it allows the trap chamber **90** to be threadably removed from the receptacle **80** and also from the net **10** to allow, for example, dead mosquitos to be removed from the trap chamber **90** that have been trapped and killed therein and/or to allow for washing of the bag **92** separate from the remainder of the net **10**. The bag **92** is made of a material that is permeable to CO_2 but that is impermeable to mosquitos (e.g., all mosquitos or at least species of mosquitos that are a malaria vector and are prevalent in the region in which the kit of parts **600** is to be deployed). In some embodiments, the kit of parts **600** may include a plug to cover the opening of the collar **70** to prevent egress of any mosquitos from the trap chamber **90**, for example and without limitation, after a subject has awaken and while mosquitos within the trap chamber **90** remain alive.

The kit of parts **600** could be applied to the top of any conventional mosquito control net **10**, an example of which is shown in FIGS. 14-16 to convert the conventional mosquito control net **10** into a trapping mosquito

control net according to one or more of the example embodiments disclosed herein. with the collar **70** has an opening formed through a thickness thereof (e.g., aligned with the direction of assembly, as shown in FIG. 13A) that is large enough to allow mosquitos to pass therethrough without deterring the mosquitos from passing therethrough. When the collar **70** and the receptacle **80** are attached to the net **10**, they form a base to which the trap chamber **90** can be removably and/or permanently attached. In some embodiments, the collar **70** and/or the receptacle **80** (e.g., using the threads **72**, **82** thereof) is/are configured to, as they are screwed together (e.g., from opposing sides of) to be secured to the conventional net **10**, cut an opening in the top surface of the conventional net **10** and is configured to mount the receptacle **80** under (e.g., suspended vertically from) the top surface of the net **10** after being attached thereto. In some embodiments, the user will manually cut a hole in the portion of the net **10** captured within the opening of the collar **70** to remove at least a portion (e.g., all) of the net **70** exposed within the opening of the collar **70**.

In some embodiments, the kit of parts **600** comprises or consists of a receptacle **80** (e.g., having a generally frustoconical shape) that is surrounded by a trap chamber that a user is able, after cutting a hole having a suitable cross-sectional shape (e.g., round) in the top surface of the net and either sews the trap chamber into place within the conventional net or uses an adhesive that is provided on a portion of the trap chamber and/or receptacle that fixes the structure to the inside and/or outside of the top surface of the conventional net. In some embodiments, the adhesive is dispensed onto the portion of the trap chamber **92** manually by a user from a container of adhesive included in the kit of parts **600.** It is to be understood that iterations are not limited to these examples and that the receptacles could be of any shape that promotes a high mosquito capture rate and low mosquito egression rate. The receptacles can further be made from any textile type.

FIG. 15A shows another embodiment in which two kits of parts **600** are attached to a top surface of a net **10** such that the net will have a plurality of discrete trap chambers to increase the efficacy of trapping mosquitos within the assembled kit of parts **600**.

FIG. 15B schematically shows an embodiment for the assembly of the kit of parts **600** to a net **10**. According to this embodiment, the collar **70** is

positioned on a first (e.g., external) side of the net **10** and the receptacle is positioned on a second (e.g., internal) side of the net **10**, with the collar **70** and the receptacle **80** being aligned with (e.g., coaxial to) each other. The collar **70** and the receptacle **80** are threadably engaged together. The portion of the net **10** corresponding to the opening formed in the collar **70** can be removed before, at the same time, or after the collar **70** and the receptacle **80** are assembled together with the net **10** secured therebetween. Once the collar **70** and the receptacle **80** are secured to the net **10** to form a base, the trap chamber **90** is threadably engaged over the receptacle **80**, preferably in a removable manner so that mosquitos can easily be removed from the trap chamber by a user after the mosquitos contained therein are dead.

FIG. 14 shows a net **10** having a kit of parts **600** according to the subject matter disclosed herein attached to a top surface thereof to conver the net **10** from a conventional mosquito control net into a trapping mosquito control net. Such a trapping mosquito control net converted from a contentional mosquito control net may still be treated with (e.g., have applied thereto) an insecticide. As shown in FIG. 14, the net **10** is positioned over a subject **20** within the net **10** and the kit of parts **600** is advantageously attached to the net **10** in a position corresponding generally to a position above the head of the subject **20**. FIG. 16 shows mosquitos **50** being drawn into the trap chamber formed by the kit of parts **600** attached to the net **10** by the presence of CO_2 being emitted from the subject **20** within the net **10**. In some embodiments, the receptacle **80** can be made of a material that is substantially impermeable to CO_2, such that mosquitos **50** are drawn towards the mouth **85** of the receptacle **80** by the flow of CO_2 therethrough to promote passage of the mosquitos **50** into the trap chamber **90** formed by the kit of parts **600**. The general presence of CO_2 about the kit of parts **600** will draw the mosquitos **50** generally towards the kit of parts **600**.

OAPI Patent No. 17063, issued on September 1, 2014, is incorporated herein by reference in its entirety.

It will be understood that various details of the presently disclosed subject matter can be changed without departing from the scope of the presently disclosed subject matter. Furthermore, the foregoing description is for the purpose of illustration only, and not for the purpose of limitation.

CLAIMS

What is claimed is:

1. A mosquito control net comprising:

an occupant chamber; and

a trap chamber disposed on the occupant chamber,

wherein the trap chamber comprises one or more openings disposed on an outer surface thereof, through which mosquitos enter the trap chamber.

2. The mosquito control net of claim 1, wherein the trap chamber is disposed on one or more side surface of the occupant chamber.

3. The mosquito control net of claim 1, wherein the one or more openings are configured such that each is large enough for the mosquitos to enter the trap chamber, but small enough to inhibit the mosquito from exiting the trap chamber, and wherein fabric surrounding the occupant chamber and separating the occupant chamber from the trap chamber is substantially impermeable to the mosquitos.

4. The mosquito control net of claim 1, wherein each of the one or more openings has a three-dimensional receptacle attached thereto, optionally each of the one or more receptacles having a frustoconical, conical, square, rectangular, hemispherical, or triangular shape.

5. The mosquito control net of claim 1, wherein the one or more openings are in a form of a funnel disposed on an outer surface of the trap chamber, and wherein the funnels are disposed such that a larger diameter edge is attached to the outer surface, sides, or both of the trap chamber.

6. The mosquito control net of claim 5, wherein each funnel comprises at least one of a woven fabric, a non-woven fabric, formed fabric, braid, lace, or a formed non-fabric material.

7. The mosquito control net of claim 5, comprising a plurality of funnels.

8. The mosquito control net of claim 7, wherein the plurality of funnels are arranged along and centered about a longitudinal axis of the mosquito control net.

9. The mosquito control net of claim 8, wherein each of the plurality of funnels has an inlet, which is attached to one of the one or more openings, and an outlet, which extends within the trap chamber.

10. The mosquito control net of claim 9, wherein the inlet is larger than the outlet for each of the funnels.

11. The mosquito control net of claim 1, wherein the trap chamber comprises a sealable opening for removing dead mosquitos.

12. The mosquito control net of claim 1, wherein the trap chamber covers an entire upper surface of the occupant chamber.

13. The mosquito control net of claim 1, wherein the trap chamber occupies a partial region of an upper surface of the occupant chamber.

14. A method for preventing mosquito bites for a subject, the method comprising:

providing the mosquito control net of claim 1; and

occupying, by the subject, the mosquito control net of claim 1.

15. The method of claim 14, comprising emptying the trap chamber of mosquito carcasses and/or washing the mosquito control net with water.

16. A method of making a mosquito control net, the method comprising:

providing a first textile configured as an occupant chamber;

providing a second textile configured as a trap chamber;

forming one or more openings in the second textile; and

attaching the trap chamber to an outer surface of the occupant chamber.

17. The method of claim 16, wherein the one or more openings are configured as receptacles formed by stamping.

18. The method of claim 16, wherein the occupant chamber and/or the trap chamber are constructed at least in part from a textile that is substantially impermeable to carbon dioxide.

19. A kit of parts for a mosquito exclusion net, comprising one or more of the mosquito control nets of claim 1 and a container for the one or more mosquito control nets.

20. A kit of parts for converting a conventional mosquito control net into a trapping mosquito control net, the kit of parts comprising a receptacle surrounded by a bag, wherein the bag is substantially impermeable to mosquitos, wherein the receptacle and/or the bag are configured for attachment to a top surface of the conventional mosquito control net to form a trap compartment.

21. The kit of parts of claim 20, comprising a screw lid having an opening of sufficiently large diameter to allow mosquitos to pass therethrough, wherein the receptacle is configured for attachment to the screw lid to extend within the trap compartment formed by the bag.

22. The kit of parts of claim 21, wherein the screw lid is configured to cut an opening in the top surface of the conventional mosquito control net as the screw lid is threadably attached to the top surface of the conventional mosquito control net.

23. The kit of parts of claim 20, wherein the receptacle and/or the bag comprise an adhesive region by which the receptacle and/or the bag are configured for adhesive attachment to an outer surface of the conventional mosquito control net for conversion into the trapping mosquito control net.

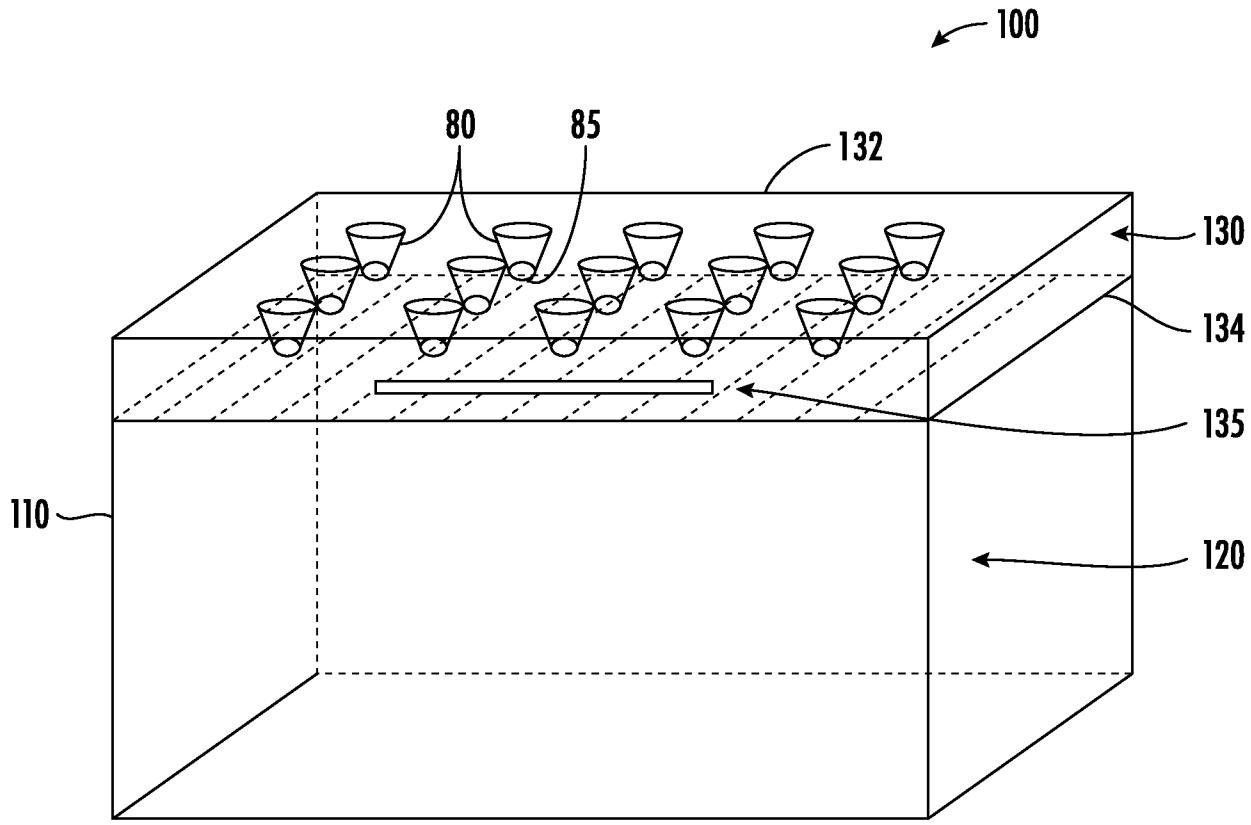


FIG. 1

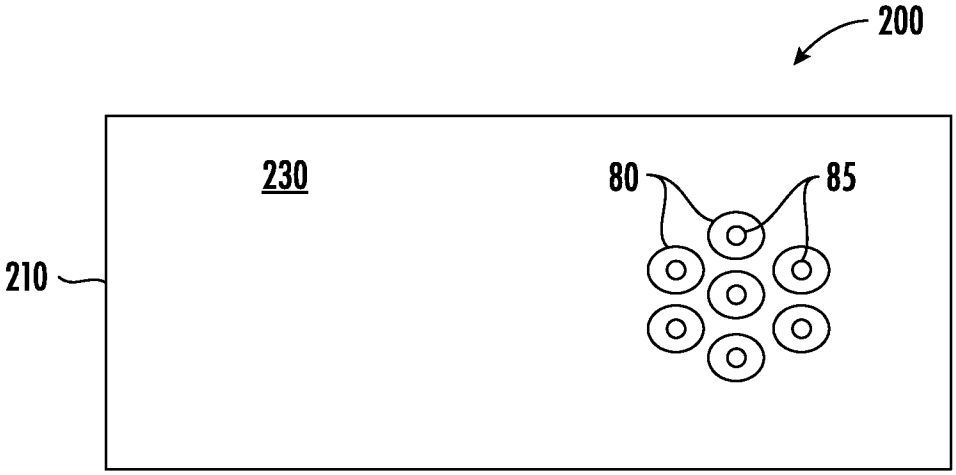


FIG. 2A

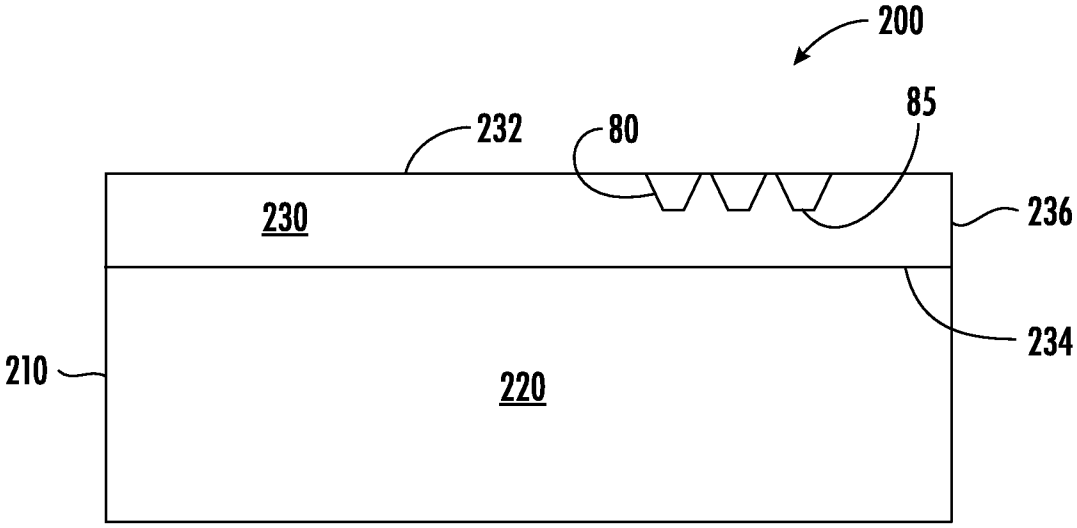


FIG. 2B

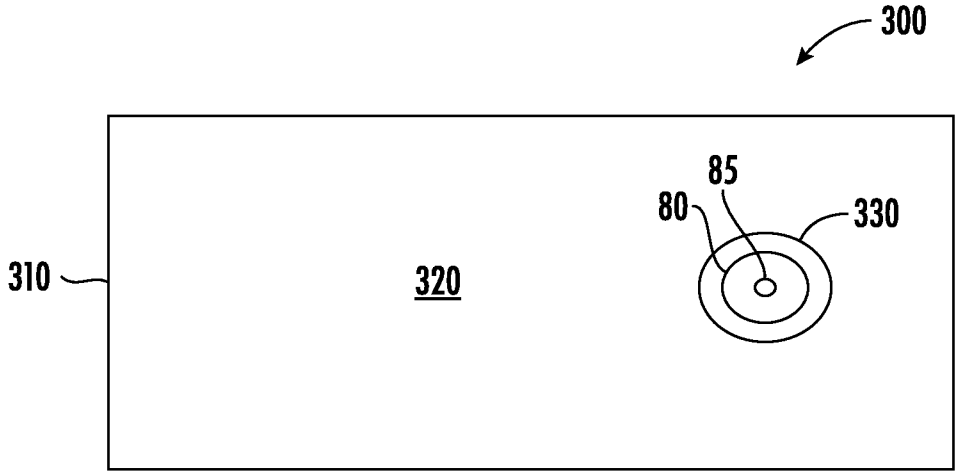


FIG. 3A

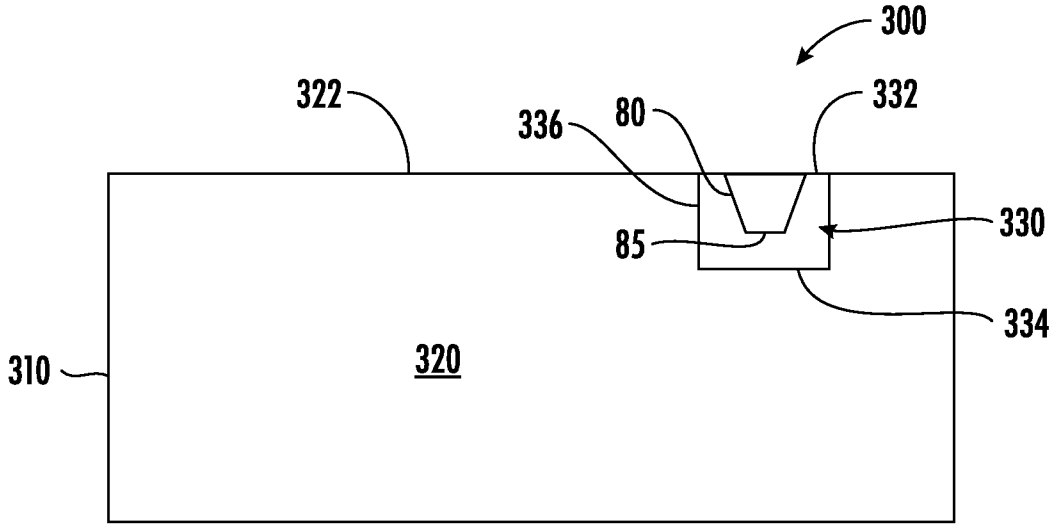


FIG. 3B

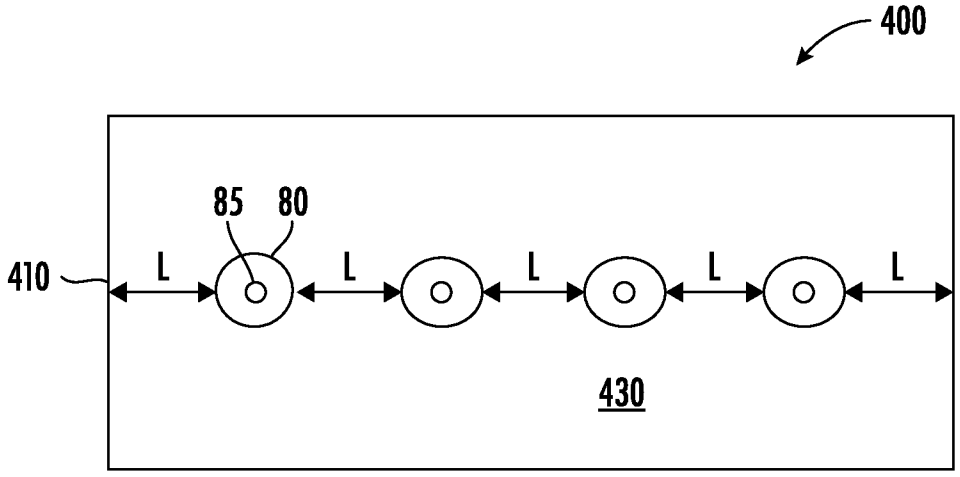


FIG. 4A

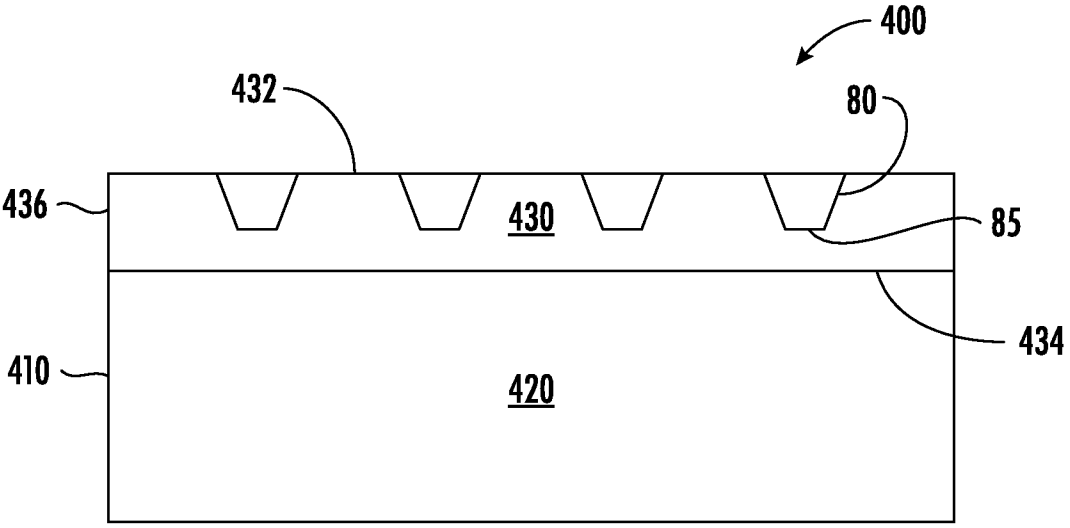


FIG. 4B

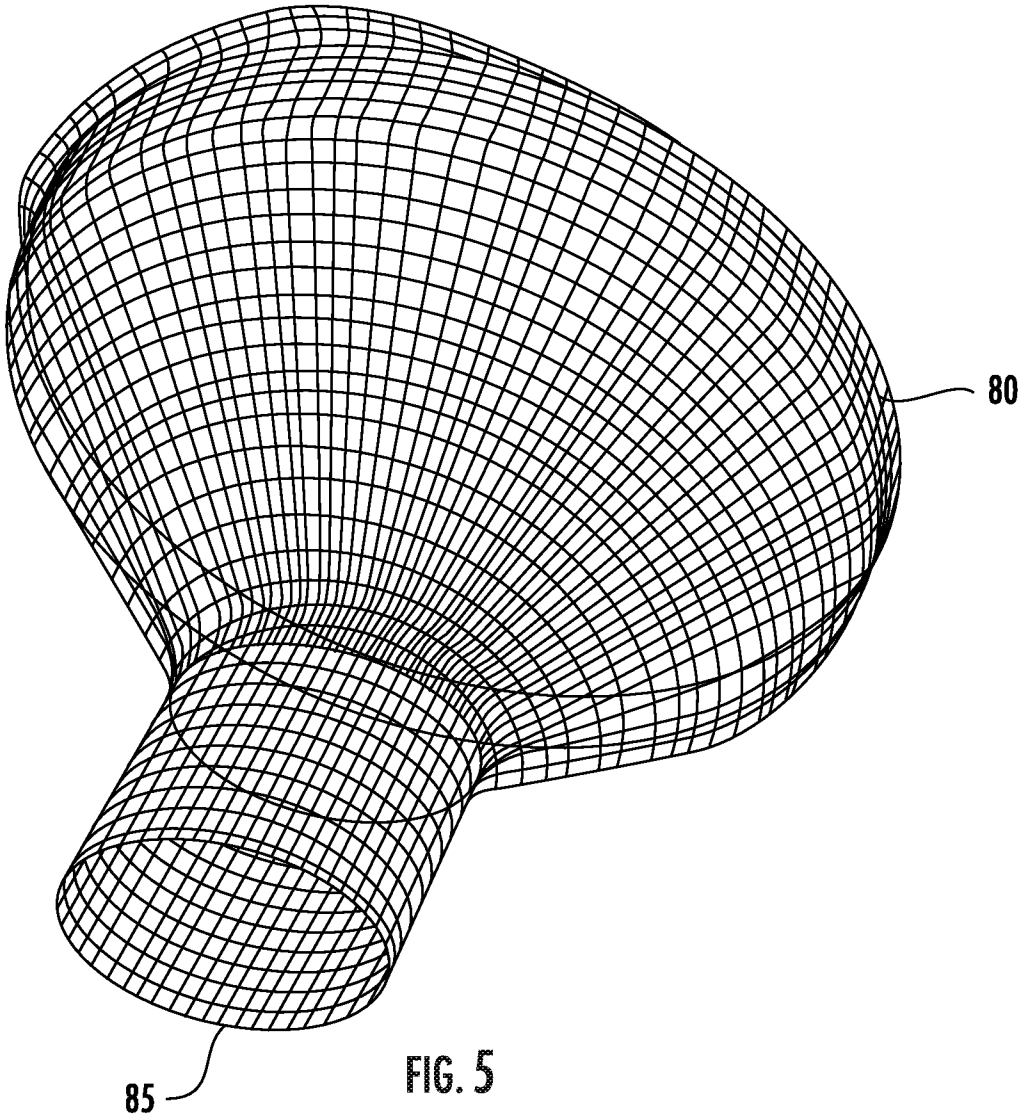


FIG. 5

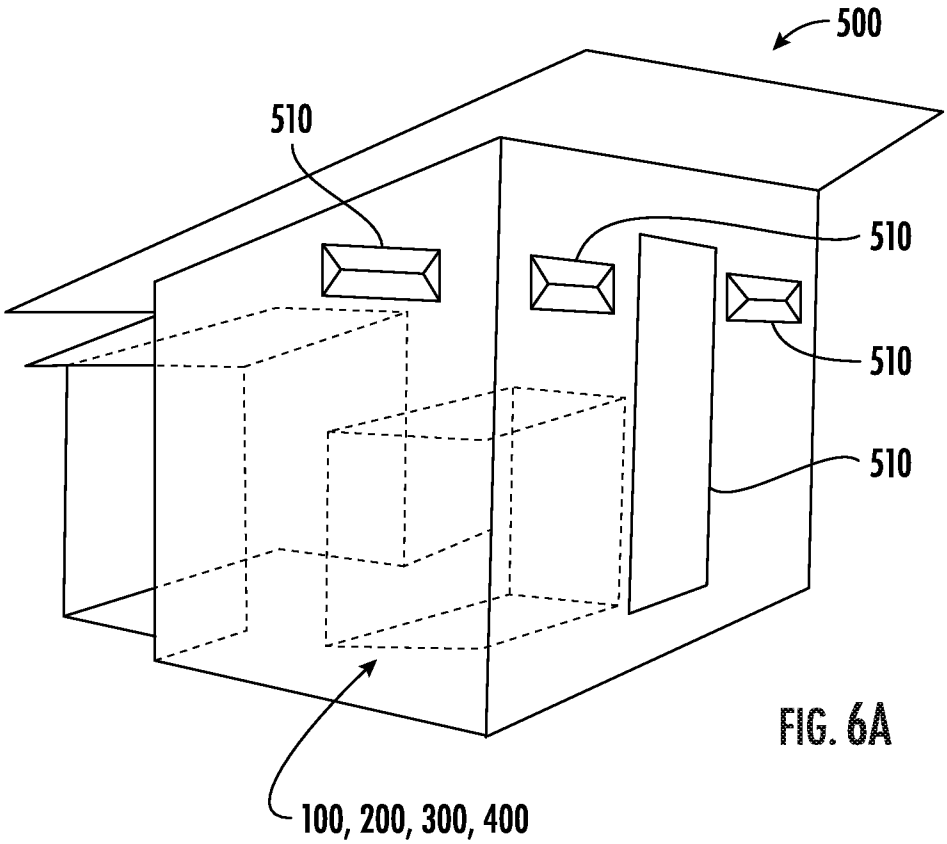


FIG. 6A

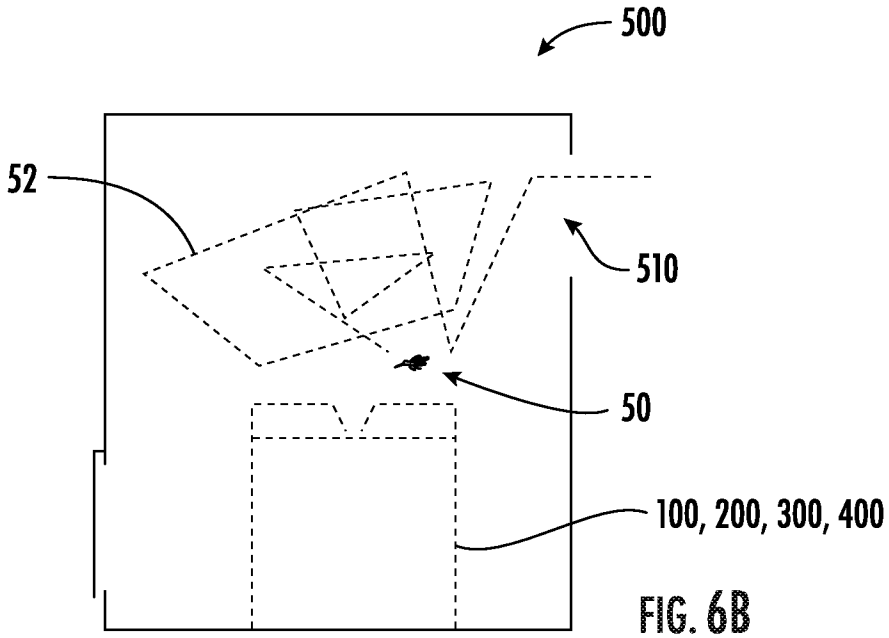


FIG. 6B

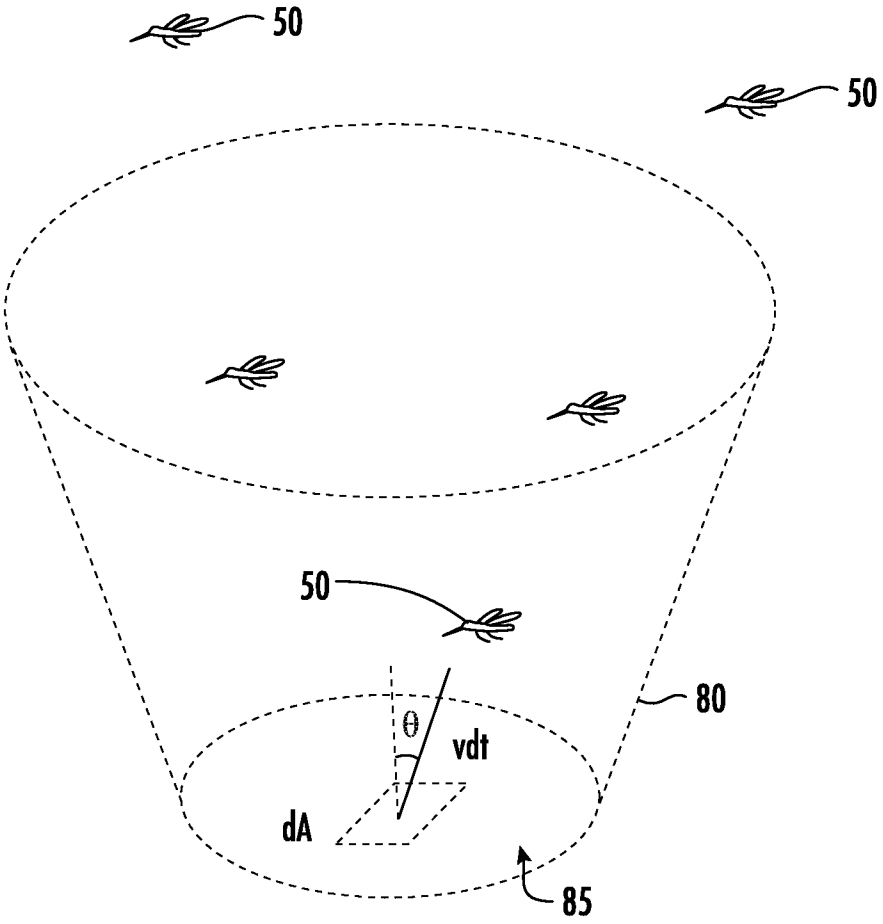


FIG. 7

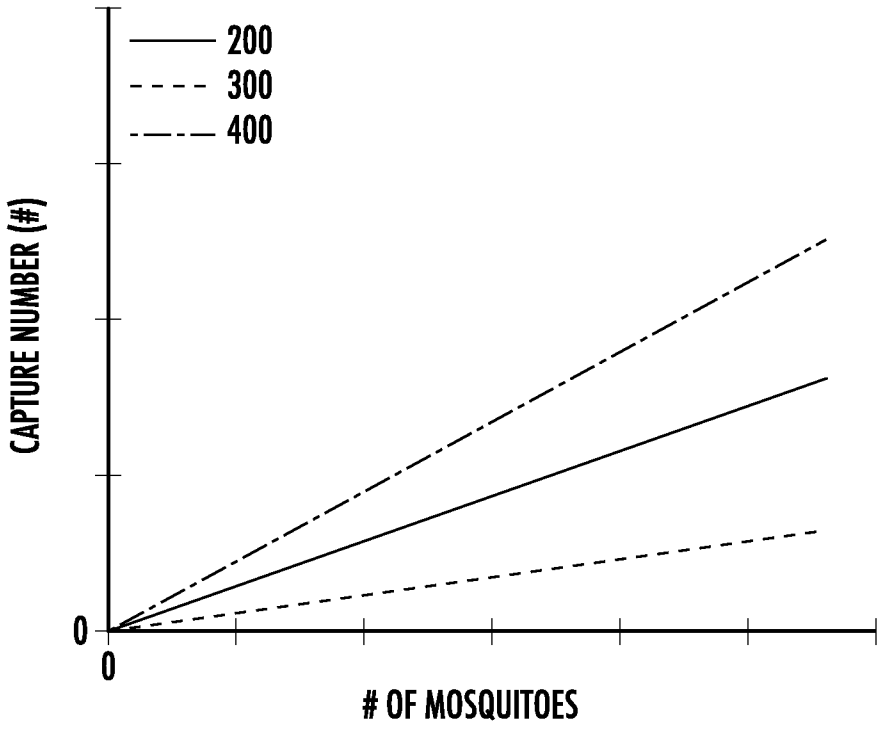

200
300
400
CAPTURE NUMBER (#)
0
0
OF MOSQUITOES

FIG. 8A

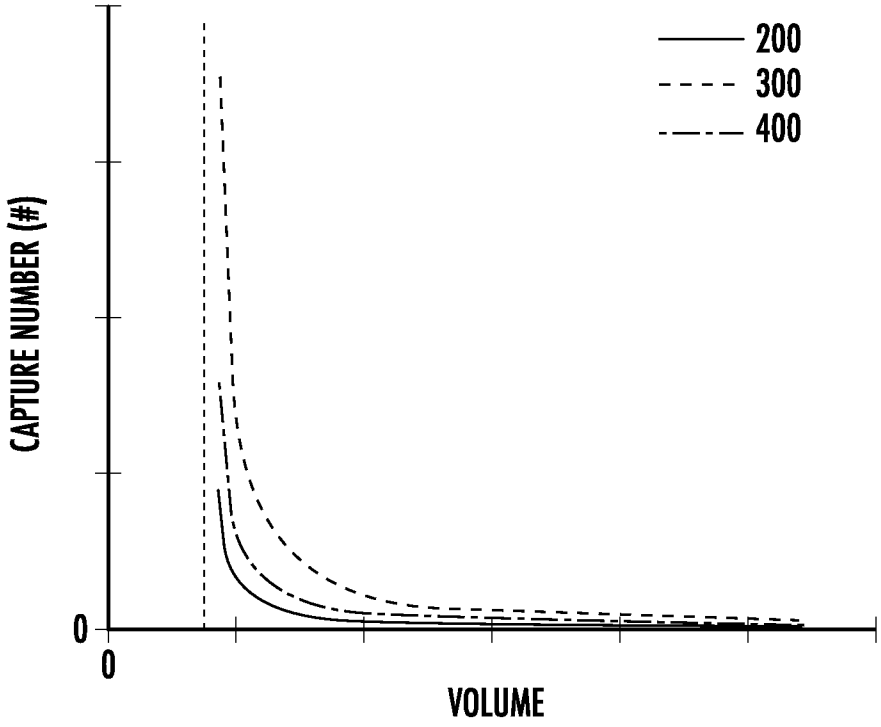

200
300
400
CAPTURE NUMBER (#)
0
0
VOLUME

FIG. 8B

			ENTRY #		EXIT RATE (%)		KILLING RATE (%)	
VARIABLE	# OBS.	TOTAL CAUGHT	MEAN*	STD DEV.	MEAN*	STD DEV.	MEAN*	STD DEV.
CONTROL	14	53	3.8A	3.0	77.2A	29.4	14.4A	18.0
200	14	139	9.9B	10.2	47.3B	27.8	39.1B	30.6
300	14	175	12.5B	10.6	45.7B	30.6	39.9B	26.1
400	14	156	11.1B	8.3	26.8B	20.7	61.9C	22.1

*VALUES NOT SHARING THE SAME LETTERS ARE STATISTICALLY SIGNIFICANT
#OBS.: NUMBER OF OBSERVATIONS
STD DEV.: STANDARD DEVIATION

FIG. 9

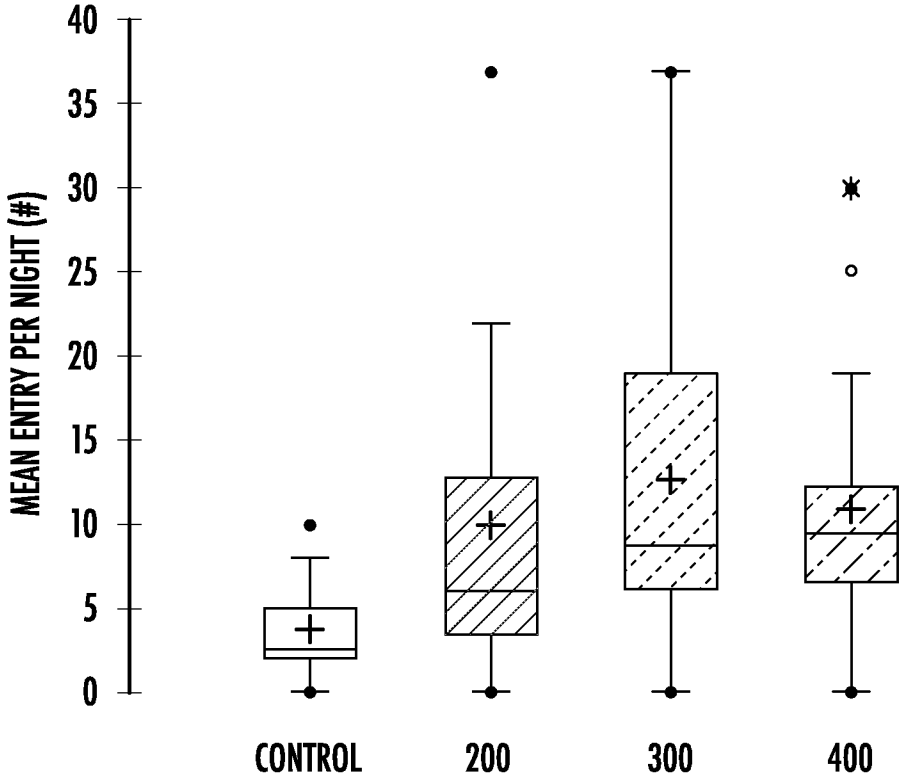


FIG. 10A

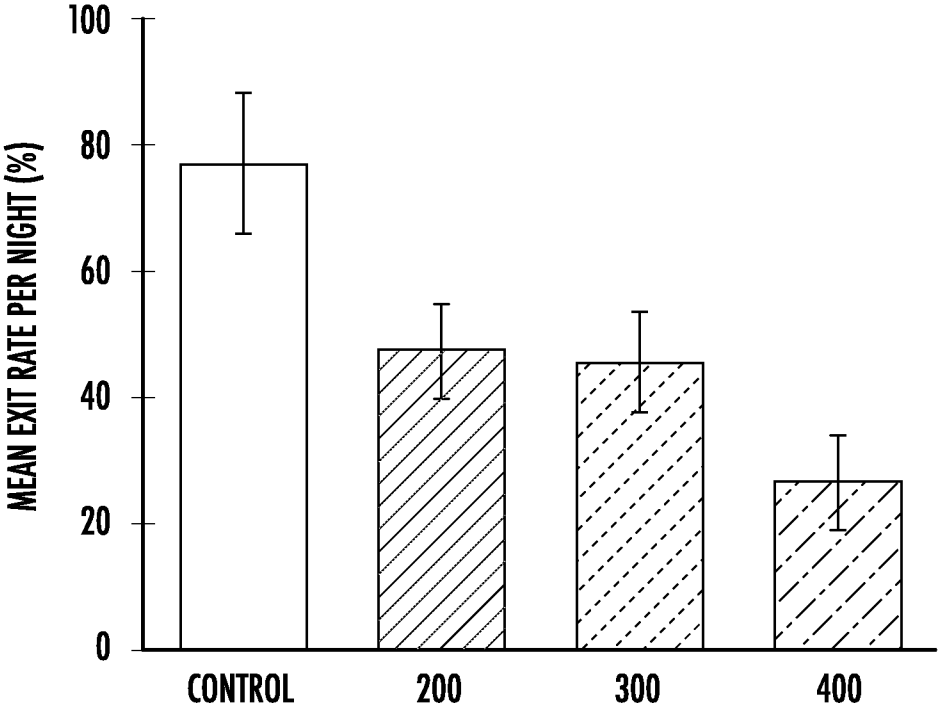


FIG. 10B

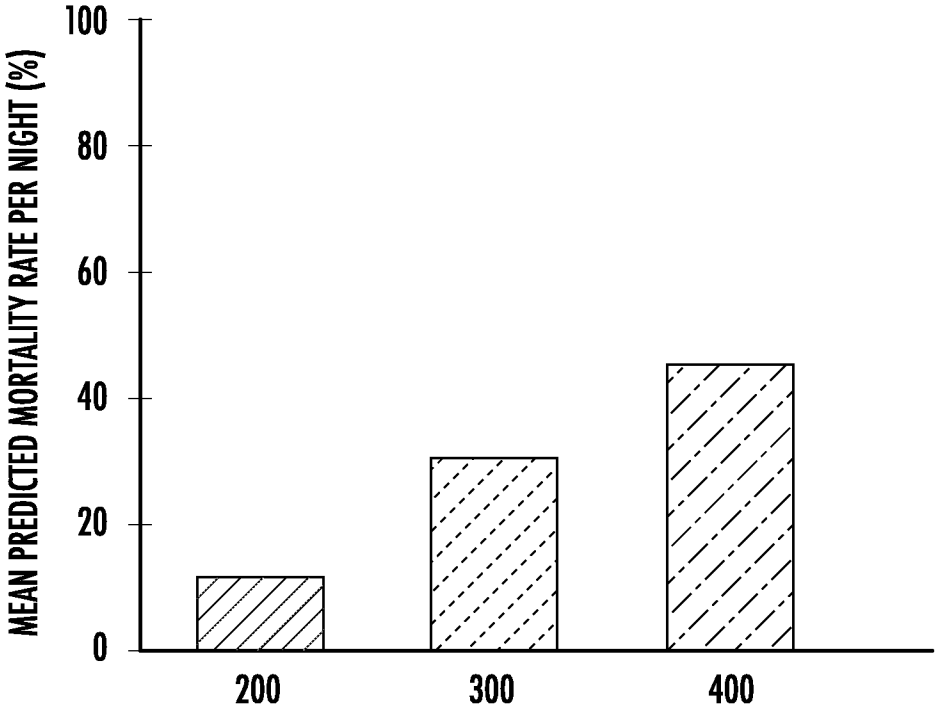


FIG. 10C

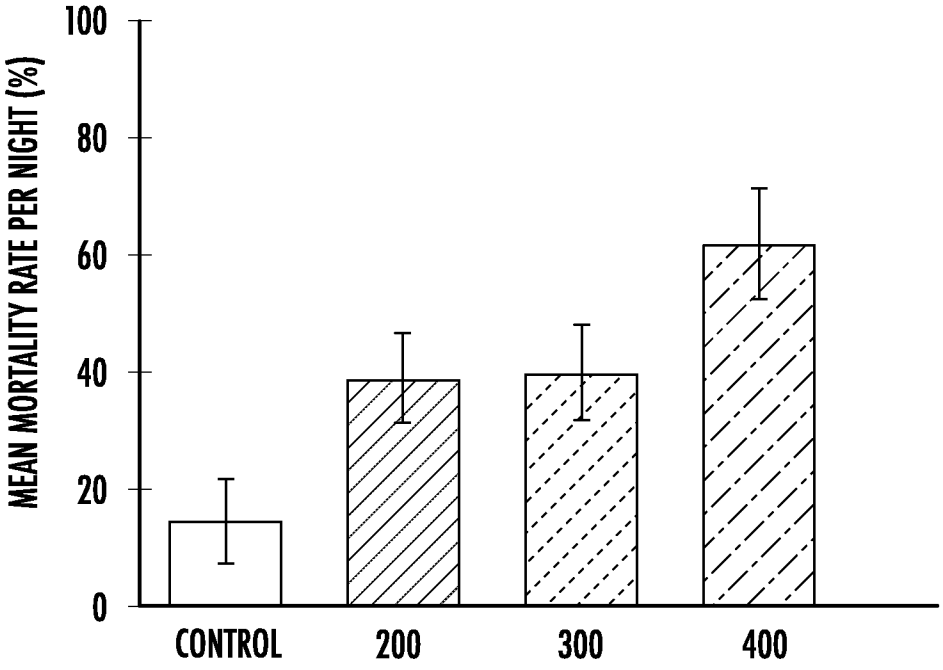


FIG. 10D

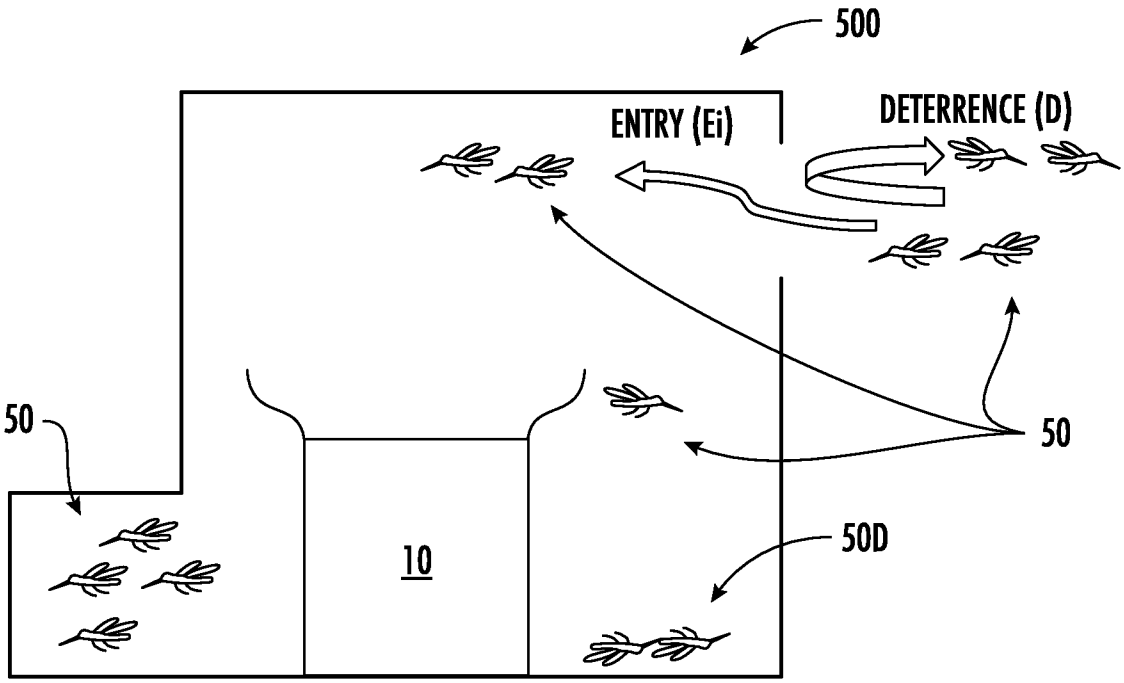


FIG. 11A
PRIOR ART

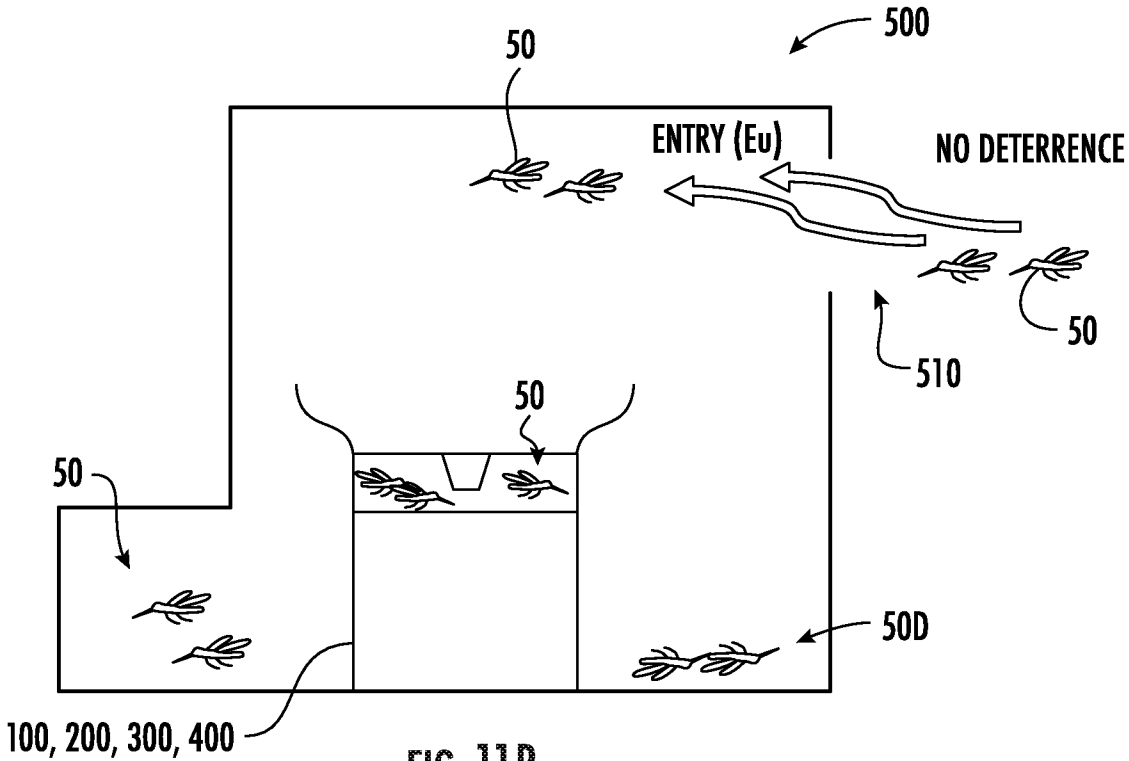


FIG. 11B

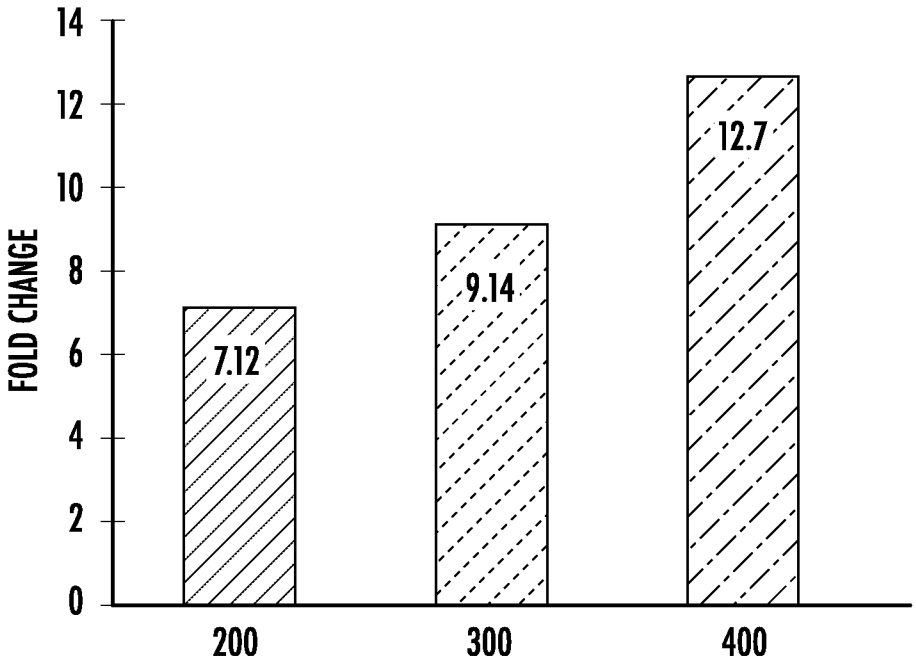


FIG. 12

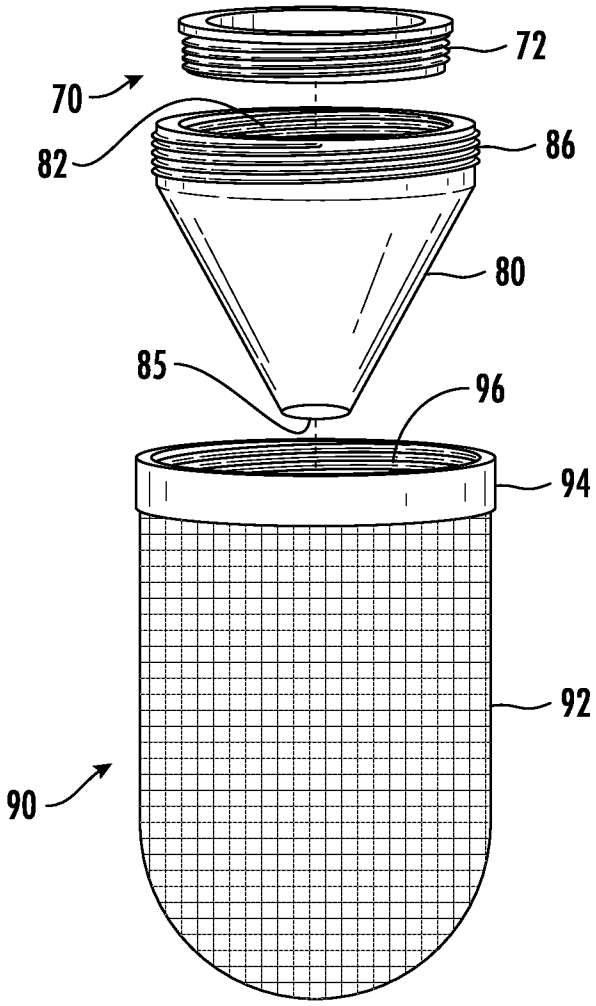


FIG. 13A

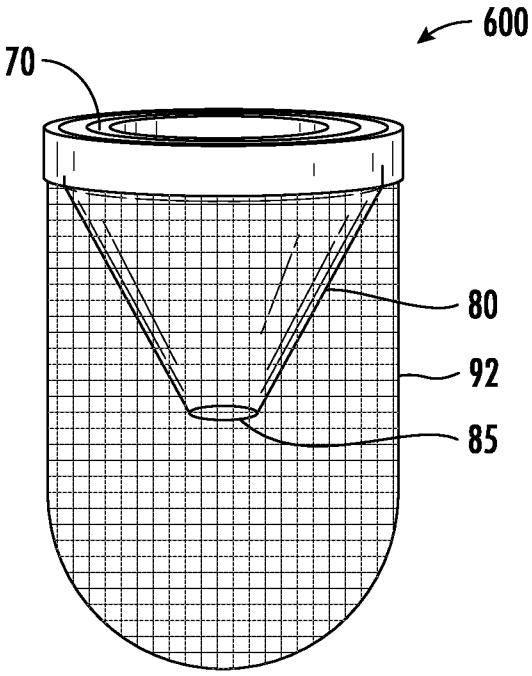


FIG. 13B

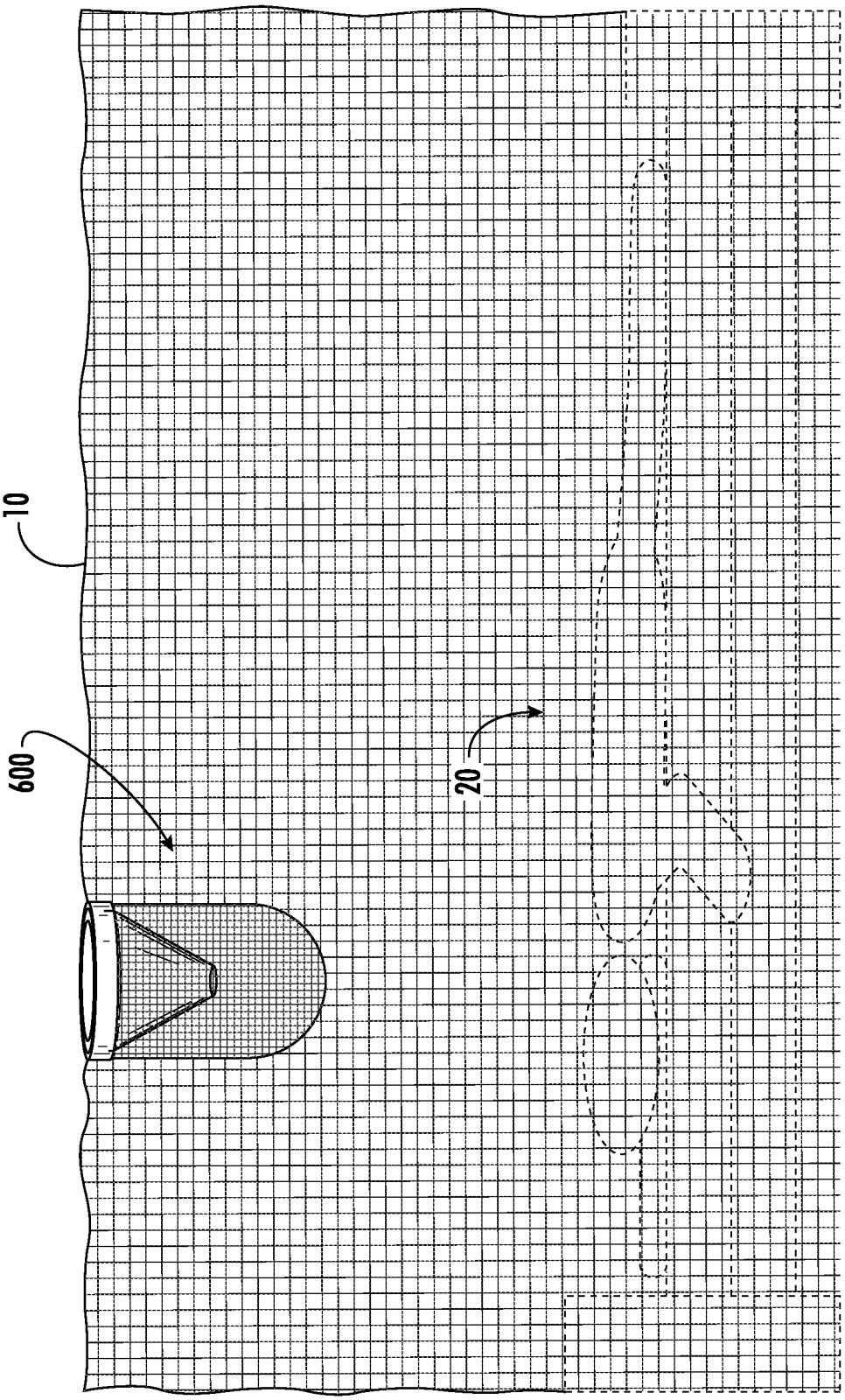


FIG. 14

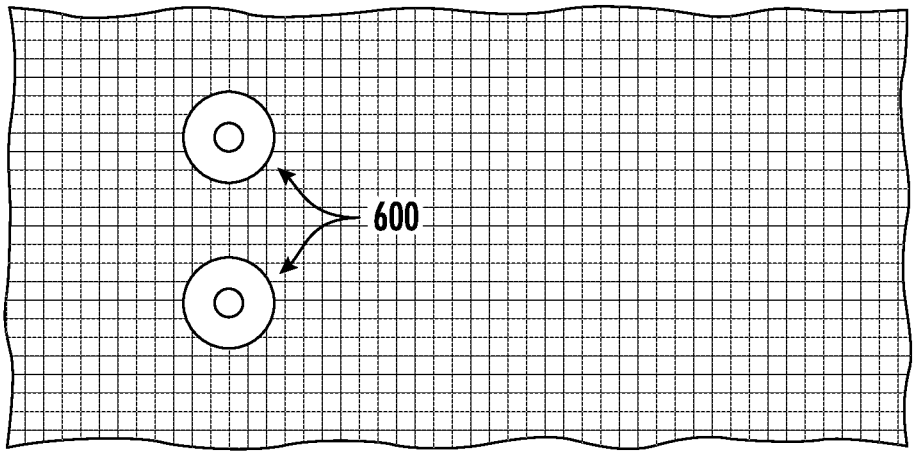


FIG. 15A

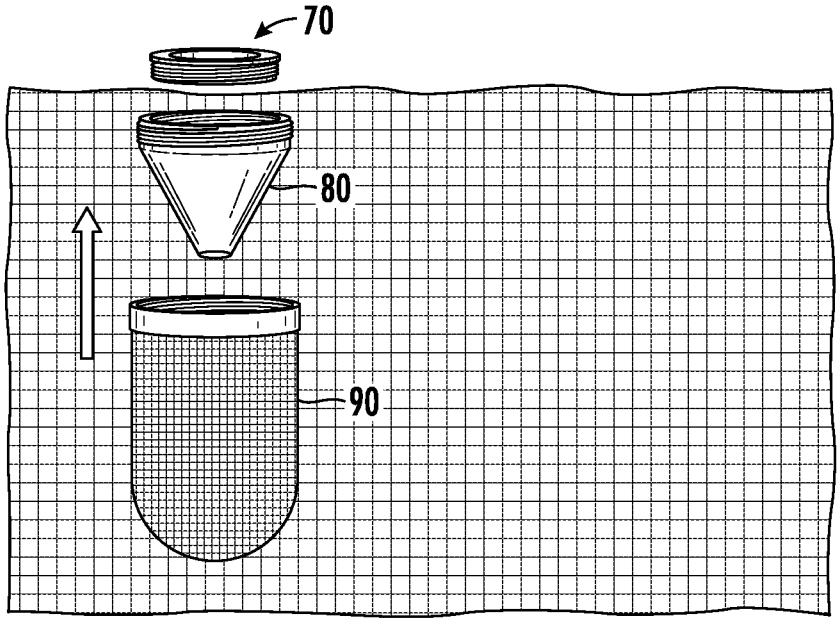


FIG. 15B

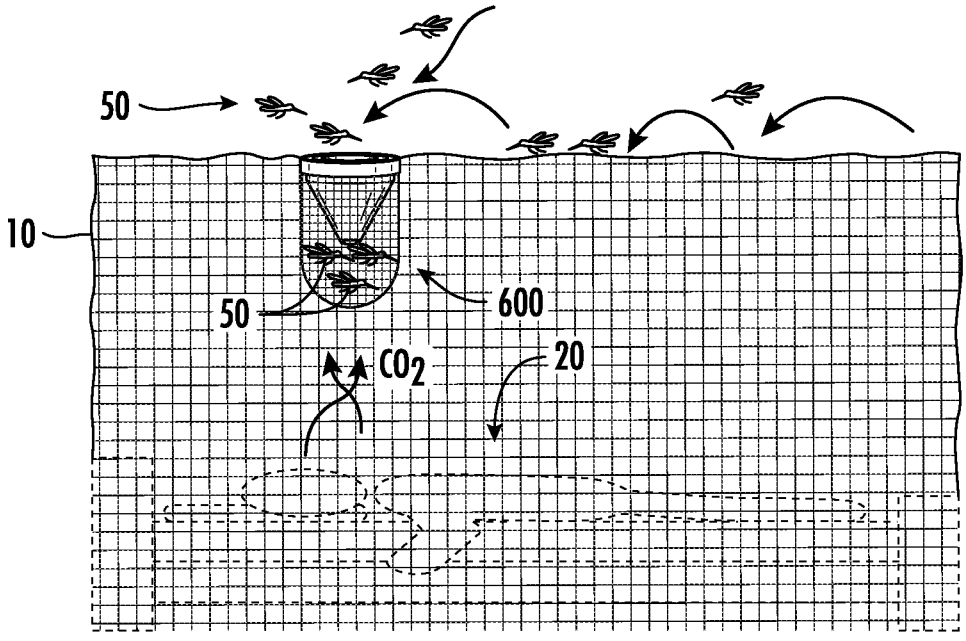


FIG. 16

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2020/045184

A. CLASSIFICATION OF SUBJECT MATTER

A01M 1/10(2006.01)i, A01M 1/02(2006.01)i, A01M 1/20(2006.01)i

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A01M 1/10; A47C 29/00; A47C 2900; A47D 13/00; A47G 9/00; B62B 9/14; E04H 1502; A01M 1/02; A01M 1/20

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Korean utility models and applications for utility models
Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
eKOMPASS(KIPO internal) & Keywords: mosquito net, trap, funnel, carbon dioxide, detach

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2016-036262 A1 (MOUHAMADOU, CHOUABOU SEDOU) 10 March 2016 pages 1-4 and figures 1-3	1-17
Y		18-23
Y	KR 10-2016-0124337 A (EWHA UNIVERSITY - INDUSTRY COLLABORATION FOUNDATION) 27 October 2016 paragraphs [0039]-[0044] and figure 1	18
Y	JP 2000-159122 A (SASAKI, REIKO) 13 June 2000 column 1, line 7 - column 2, line 9 and and figures 1-3	19
Y	US 6263894 B1 (LAMANTIA, MARK) 24 July 2001 column 2, line 35 - column 4, line 54 and figures 1-4	20-23
A	CN 108967390 A (WANG, WEI) 11 December 2018 paragraphs [0039]-[0044] and figure 1	1-23

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"D" document cited by the applicant in the international application
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents,such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 November 2020 (16.11.2020)	**17 November 2020 (17.11.2020)**

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Korean Intellectual Property Office
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Authorized officer
BAHNG SEUNG HOON
Telephone No. +82-42-481-5560

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/US2020/045184

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2016-036262 A1	10/03/2016	None	
KR 10-2016-0124337 A	27/10/2016	KR 10-1693105 B1	05/01/2017
JP 2000-159122 A	13/06/2000	None	
US 6263894 B1	24/07/2001	AU 782954 B1 CA 2311260 A1	15/09/2005 09/12/2000
CN 108967390 A	11/12/2018	None	

International Journal of
Environmental Research and Public Health

MDPI

Review

Biology, Systematics, Microbiome, Pathogen Transmission and Control of Chiggers (Acari: Trombiculidae, Leeuwenhoekiidae) with Emphasis on the United States

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[2] Comparative Medicine Institute, North Carolina State University, Raleigh, NC 27695, USA
* Correspondence: loganathan_ponnusamy@ncsu.edu

Abstract: Chiggers are the larval stage of Trombiculidae and Leeuwenhoekiidae mites of medical and veterinary importance. Some species in the genus *Leptotrombidium* and *Herpetacarus* vector *Orientia* species, the bacteria that causes scrub typhus disease in humans. Scrub typhus is a life-threatening, febrile disease. Chigger bites can also cause dermatitis. There were 248 chigger species reported from the US from almost every state. However, there are large gaps in our knowledge of the life history of other stages of development. North American wide morphological keys are needed for better species identification, and molecular sequence data for identification are minimal and not clearly matched with morphological data. The role of chiggers in disease transmission in the US is especially understudied, and the role of endosymbionts in *Orientia* infection are suggested in the scientific literature but not confirmed. The most common chiggers in the eastern United States were identified as *Eutrombicula alfreddugesi* but were likely misidentified and should be replaced with *Eutrombicula cinnabaris*. Scrub typhus was originally believed to be limited to the Tsutsugamushi Triangle and the chigger genus, *Leptotrombidium*, but there is increasing evidence this is not the case. The potential of *Orientia* species establishing in the US is high. In addition, several other recognized pathogens to infect humans, namely Hantavirus, *Bartonella*, *Borrelia*, and *Rickettsia*, were also detected in chiggers. The role that chiggers play in these disease transmissions in the US needs further investigation. It is possible some of the tick-borne diseases and red meat allergies are caused by chiggers.

Keywords: chiggers; biology; systematics; microbiome; pathogen transmission; control

check for updates

Citation: Chen, K.; Roe, R.M.; Ponnusamy, L. Biology, Systematics, Microbiome, Pathogen Transmission and Control of Chiggers (Acari: Trombiculidae, Leeuwenhoekiidae) with Emphasis on the United States. *Int. J. Environ. Res. Public Health* **2022**, *19*, 15147. https://doi.org/10.3390/ijerph192215147

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1. Introduction

Chiggers are the larval stage of the families Trombiculidae and Leeuwenhoekiidae mites (Phylum Arthropoda, Class Arachnida, Order Trombidiformes, Superfamily Trombidioidea, and Family Trombiculidae, Ewing, 1929 and Family Leeuwenhoekiidae, Womersley, 1945. They are also called red bugs, harvest mites, berry bugs, scrub-itch mites in English-speaking countries, and sand mites in China. Chiggers are of medical and veterinary importance. Some species in the genus *Leptotrombidium* and *Herpetacarus* [1,2] are known to vector a Gram-negative bacterium, *Orientia* species (formerly in the genus *Rickettsia*), causing the disease, scrub typhus, in humans. Scrub typhus is a life-threatening, febrile disease. During the Second World War, a large number of scrub typhus patients were found in the Asia-Pacific region [3], which resulted in greater attention and research on all aspects of the disease. Humans are only an occasional host for chiggers. Chiggers parasitize many different vertebrates, including reptiles, amphibians, birds, and mammals, and were reported to be significant pests for free-range turkeys in the US [4]. Their bite frequently results in dermatitis (trombiculosis or trombiculiasis).

Publications of chigger studies in the US were searched on the Web of Science on 3 March 2022 (Table S1), showing 115 papers of which 83 were directly on chiggers. The

number of publications on chiggers in the US is shown in Figure 1. Publications were summarized and shown in Table S2. The publications mainly focused on taxonomy (identification methods), ecology, and reports of the discovery of chiggers on various hosts in the US. Chigger research in the US started in the 1910s. The number of publications gradually increased from 1920 to 1979 followed by a drop in the 1980s. From 1990 till now, the number of publications per decade on this topic was 13–16. However, these data do not include all of the existing published papers, especially the older papers. For example, as stated in the world checklist of Trombiculidae and Leeuwenhoekiidae from Nielsen et al. [5], H.E. Ewing described 64 new chigger species from 1913 to 1950. Nielsen et al. listed 23 Ewing's publications and five were marked as cited by other researchers. Based on our search results, there were only 10 papers published by Ewing that were available on the Web of Science, but additional papers were available at our institutional library.

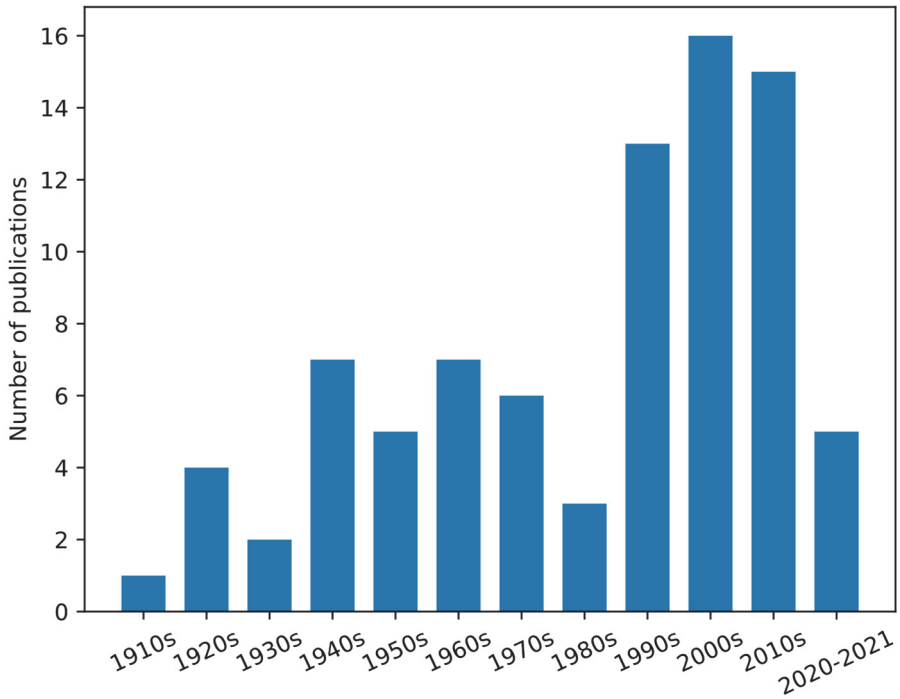


Figure 1. Bar chart of publications of chiggers in the US. Publications were searched through the Web of Science on 3 May 2022. The search strategy is in Table S1).

It is challenging to find papers about chigger studies in the US published 50–90 years ago. Even for the publications that can be found on the Web of Science, some are not easily accessible for review. Publications after the 1990s were better documented. It is clear that chiggers in the US are greatly understudied in general and especially as compared to other vector important arthropods like mosquitoes, ticks, lice, fleas, and filth flies. The goal of this paper was to review the biology, systematics, microbiome, pathogen transmission and control of chiggers with emphasis when possible on what we know about them in the US.

2. Chigger Biology

2.1. Life Cycle

The life cycle of chigger mites is comprised of seven stages: egg, quiescent prelarva (also called deutovum), parasitic larva, quiescent protonymph, soil free-living deutonymph, quiescent tritonymph and soil free-living adult (Figure 2). The two active post larval stages, deutonymph and adult, have eight legs, live in soil, and are predators feeding on arthropod eggs and other life stages of small, soil-dwelling insects and other arthropods. The parasitic larval stage, so-called chiggers, have six legs. Unengorged chiggers are about 0.2 mm long and deep-red in color, while engorged chiggers are about 0.4 mm long and pale yellow [6,7].

Chiggers do not burrow into skin and feed on blood as many may think. Instead, chiggers feed on saliva digested lymph and skin cells. They insert their chelicerae into the skin and secrete saliva containing proteolytic enzymes into the skin. The epidermal and cutaneous host tissue is liquefied, and the liquid is consumed. As the feeding progresses, a hard tube-like structure, called a stylostome, is formed by the salivary secretions and the host immune system responds to the foreign enzymes in the chigger saliva [8]. The stylostome functions as a straw, allowing the chiggers to consume the exo-digested skin. The saliva secretion and stylostome formation from feeding cause an inflammatory dermal reaction in the host [9], which is called trombiculiasis. Chiggers will attach to a host and feed for 1–12 d if not scratched off [10–12], the duration variable depending on the host species. After engorgement, chiggers drop naturally from the host to the soil and become quiescent.

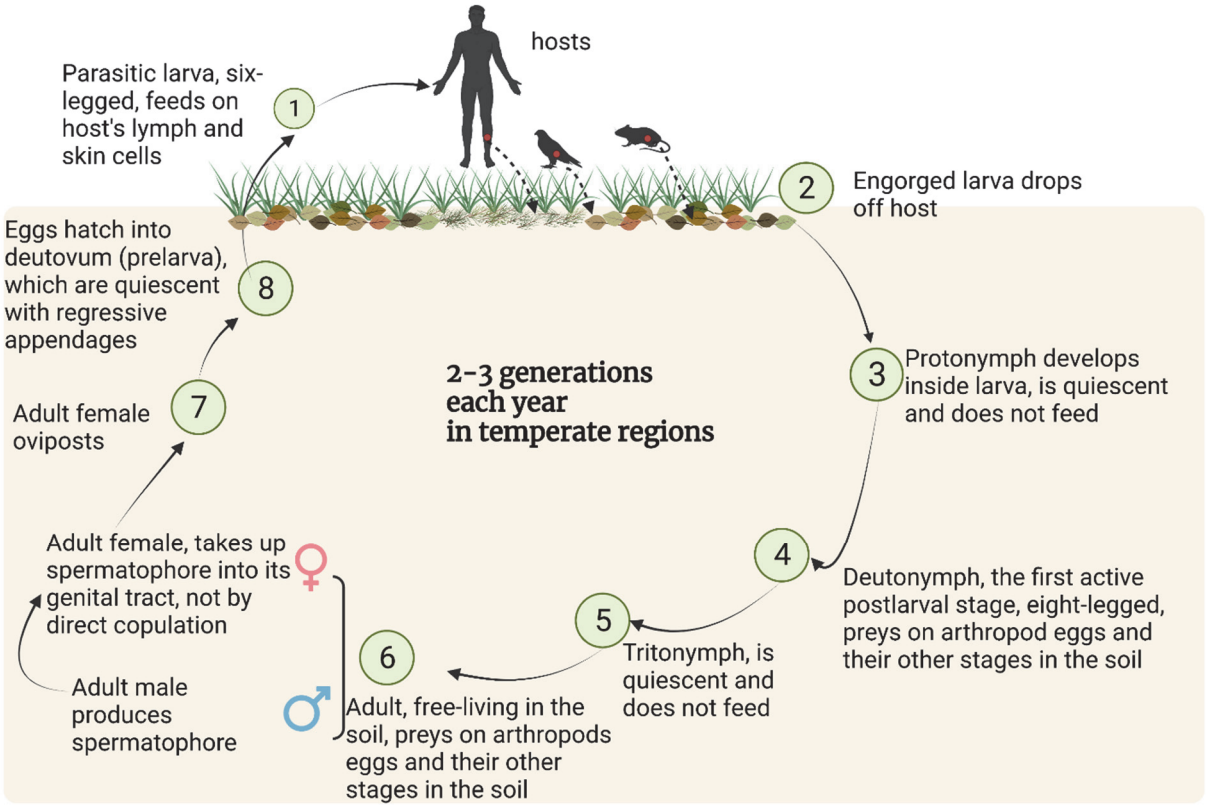


Figure 2. The typical life cycle of chiggers. Created with BioRender.com. Data sources: [11,13,14].

Overall, the available data for the developmental time for each stage both in the field and lab are limited. In optimal lab conditions (25–30 °C, 100% relative humidity), a generation of *L. deliense* is 59–135 days, with an 89 days average [13]. The development time of a complete generation for boreal species can take anywhere from 150 to 400 days [14]. In the field, local climate conditions will obviously affect the developmental time since chiggers are ectotherms. Wharton and Fuller [15] reported *E. alfreddugesi* produced one to two generations a year in Ohio, three generations in North Carolina and developed year-round in Florida, US. Direct copulation between males and females has never been observed. The adult female is inseminated by making contact with a spermatophore deposited by males in the habitat that they share [16]. The male spermatophore is made up of a flexible stalk (about 5 µm thick and 45 µm tall) that is linked to the substrate and bears a sperm sac at the distal end [11,16]. When the female encounters the spermatophore, she will walk over the spermatophore with her genital plates fully opened and pick up the sperm sac. Lipovsky et al. [16] observed that chigger females could acquire multiple

spermatophores. Females normally undergo one to three oviposition cycles with gaps between them throughout their lives [14].

2.2. Hosts and Habitat

The host spectrum of chiggers is broad. For example, *E. alfreddugesi*, the most widespread chigger species in the US, were found to parasitize reptiles, amphibians, birds and mammals. Walters et al. [17] listed that 145 species of reptiles and amphibians, 129 species of birds, and 225 species of mammals in the US were reported to be parasitized by chiggers. Some chigger species or genera were only reported parasitizing a particular host group. For example, almost all *Neoschoengastia americana* parasitize birds [17] and this species was reported to be a significant pest of turkeys in the US [4]. Chiggers in the genus *Hannemania* primarily fed on amphibians [17]. However, the preference of chiggers to a specific host species is low. Chiggers are habitat-specific rather than host-specific. Chiggers are known to have preferences for a certain local habitat, where they attack and feed on all or the majority of the vertebrate species in the same location [14,17–19]. The low host specificity of the chigger and broad host range may enhance their chances of contacting humans, as well as the spread of scrub typhus across diverse hosts. Scrub typhus so far has not been reported in the US (discussed in more detail later).

Chiggers prefer moist areas with overgrown vegetation [15], like abandoned plantations, tall grass, and river banks. Chiggers are found to be most abundant in early summer in temperate regions when the vegetation is heaviest. Clopton and Gold [20] reported the abundance of *E. alfreddugesi* peaked in late June to early July. In the summer, adults were most common near the soil surface, and in the fall and winter, they were found deeper in the soil [15]. Shatrov and Kudryashova [14] reported the eggs of some boreal chigger species can diapause for up to 400 d. This suggests they have temperature preferences likely related to their life cycle and survival when environmental conditions are not favorable for development.

2.3. Collecting Methods

2.3.1. Trapping Animals

This method of collecting requires approval from Institutional Animal Care and Use Committees (IACUCs). Rodents can be live-trapped with Sherman traps (Figure 3A) placed on the ground overnight at any place where chiggers and potential hosts are expected to be found [21]. Chiggers then can be collected from rodents the next day. Sherman traps are usually baited with bird seed [22] or a peanut butter and oats mixture [23]. Ketamine hydrochloride and xylazine sulphate (1:10) are given intramuscularly to anesthetize the captured rodents [22]. Captured rodents are examined thoroughly for the presence of chiggers with particular attention to the ears, groin, foot, belly and area surrounding the genitals [23]. Removed chiggers are preserved in a vial containing 70% ethanol [22]. Similar approaches may be used for other types of animals like birds, lizards, or larger mammals. One advantage of this approach, the rodents are traveling throughout their natural geographical range foraging for food, obtaining nesting materials and mating and have a greater chance of acquiring chiggers from their habitat than other collection methods (discussed later). Additionally, the chiggers are easy to collect from the animal after trapping, since they are attached to specific locations, e.g., in the ears of mice. This approach is only effective for obtaining feeding chiggers. Since they are feeding on animals that could be infected with a human pathogen, for example, their microbiomes might reflect that of the host and not necessarily that of a free-living chigger.

2.3.2. Plate Method

Unengorged chiggers can be attracted to foreign objects of almost any kind [24], for example, a porcelain plate, plastic disk, or oilcloth [21,24]. Black plates (Figure 3B) are attractive to unfed chiggers when heated by the sun, and the black color makes it easy to see the reddish color of chiggers on the plate, especially when they are crawling. Thus, black

plates were commonly used to collect unengorged, free-living chiggers in rodent runways and near nests [25–28]. A set of black plates (10 cm by 10 cm) are laid at a possible breeding site at intervals. Each black plate is observed for any movement after a certain amount of time (e.g., 10 min). Chiggers can be collected with an ethanol-dipped pipette or small camel hair or equivalent brush and then transfer to a tube containing 70% ethanol. A hand lens helps ensure a successful transfer. Another collecting method is using an inverted white tray placed on the ground in a chigger infested area overnight; when the tray is righted the next day, free-living chiggers can be collected from what was the undersurface of the tray [21,24].

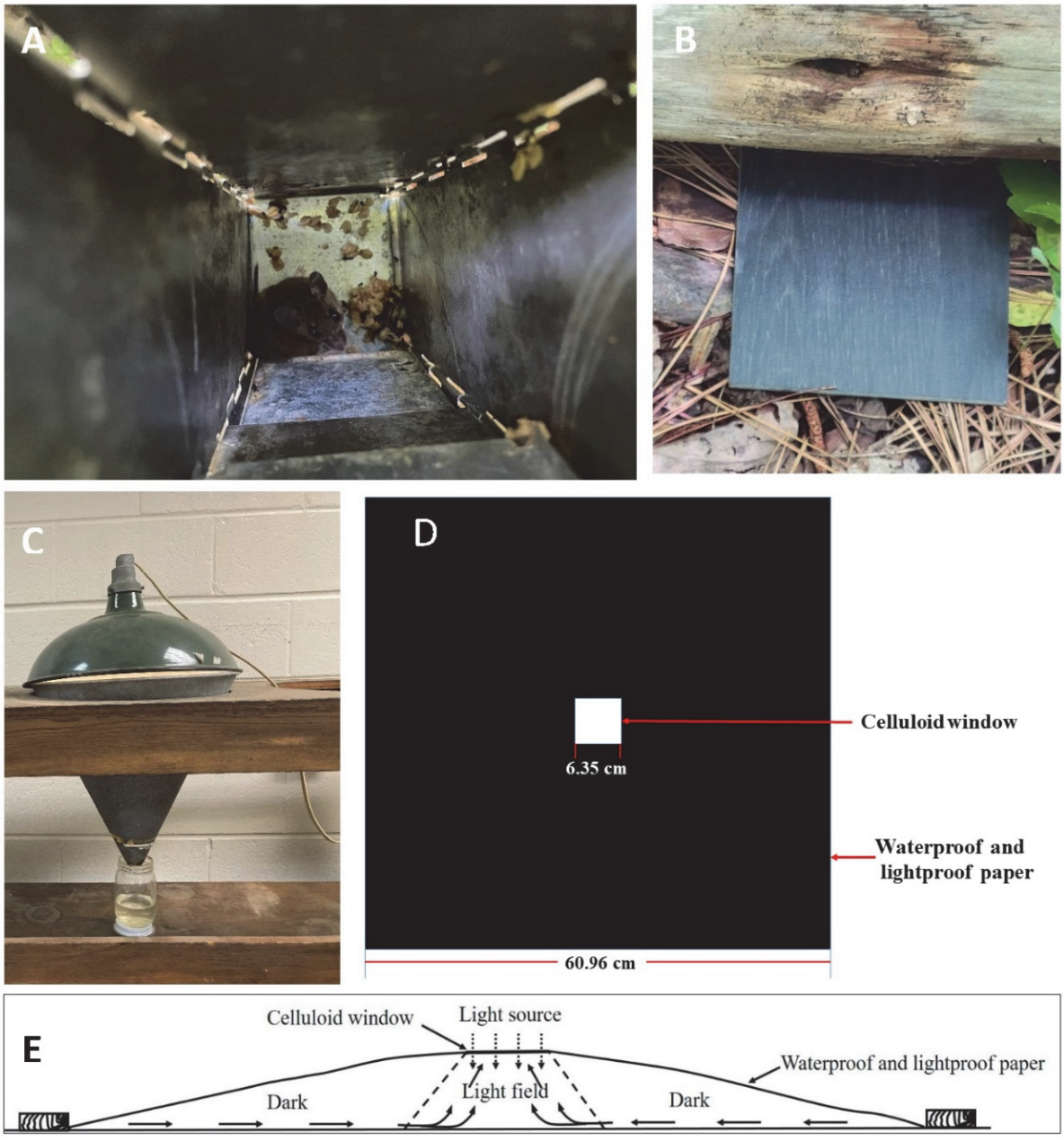


Figure 3. Chigger collecting methods: (**A**) live-trapped rodents in Sherman traps (photo credit to Reuben Garshong); (**B**) black tile on the ground; (**C**) Berlese funnel (photo credit to Steve Denning); (**D**) light trap (modified from [29]); (**E**) horizontal view of light trap, arrows inside the trap show the paths taken by chiggers (modified from [29]).

The advantages of the plate method, free-living chiggers are collected alive and in good viable condition, in case the researcher wanted to develop a laboratory colony or study the live biology, physiology or microbiome of the chiggers. The challenge with this method is finding an exact location where the chiggers are found. This is typically a random approach within a potential geographical location where chiggers are expected. Sometimes, picking a place to collect can be enhanced by incidental reports of chigger problems, frequently provided by park rangers or comments from visitors to a particular area.

2.3.3. Berlese Funnel

A Berlese funnel (Figure 3C) can be used to collect chigger mites from soil simply by placing the litter sample into the inverted funnel. The mites will crawl downward from the light shining into the top, large opening of the funnel, as the soil dries and they eventually fall into a bottom collection chamber filled with ethanol (to kill and preserve the mites) positioned below the end of the inverted funnel [18]. The challenge with this approach would be the need to collect enough soil samples to find the mites and have enough Berlese funnels to process the soil samples, since the free-living chiggers are not typically widely and evenly distributed in the landscape. Additionally, other soil animals will drop into the ethanol and will need to be separated from the free-living chiggers. If there is an interest in the external microbiome of the chigger, there would be cross contamination between animals. The chiggers collected by this method are killed by the ethanol.

2.3.4. Light Trapping

Johns [29] developed a light trap to collect unengorged chiggers in vegetation using the natural tendency for the unengorged chiggers to be positively phototropic with a preference to climb higher. The trap was made of a piece of 60.96 cm by 60.96 cm waterproof and lightproof paper with a 7.62 cm by 7.62 cm hole cut in the middle. A celluloid window measuring 8.26 cm by 8.26 cm was placed over the hole and attached to the paper with adhesive tape to make a central window frame about 6.35 cm by 6.35 cm (Figure 3D). The trap is laid in the desired location with the celluloid window in contact with the soil surface or the vegetation so the chiggers could climb from the dark field to the celluloid window (Figure 3E). The edges of the trap are secured by bricks, stones or wood pieces to hold the sheet to the ground and assure the only light entering the trap is was from the top celluloid window. When collecting the chiggers, the trap is turned over, and the chigger transferred by a wet brush onto a moistened filter paper in a collecting jar to extend the life of the chigger. This approach to keep the free living chiggers alive could also be used for the plate method of collecting (discussed earlier).

2.4. Chigger Lab Rearing

A chigger colony can be started from field-collected engorged larvae taken from trapped hosts. The larvae are allowed to detach from the host naturally to avoid killing them [30]. This will require a different IUCUC protocol for holding the wild-caught host longer than typically needed for collecting chiggers from a trapped host which are released the same day. Collecting chiggers for starting a lab colony is more challenging because the wild animals have to be maintained in environmental conditions to preserve the health and well-being of the host. For biosecurity reasons, wild hosts cannot be held in a rearing facility with other research animals because of the threat of introducing a disease.

Chiggers throughout their life cycle are maintained in 3.5 cm diameter plastic containers, 1/3 filled at the bottom with hardened plaster of Paris: charcoal (9:1), saturated with water [31]. Michener [32] also reported using fruit jars with the inner surface coated with 1/8 to 1/4 inch thickness of plaster of Paris to rear *Eutrombicula batatas* in the lab. Eggs of collembola, e.g., *Sinella curviseta* [33] or of mosquitoes, e.g., *Culex pipiens* [18], are provided ad libitum to nymphs and adults as food. Eggs before hatching are separated from the other stages by flooding the container; the eggs remain at the bottom [30]. The unfed chigger larvae are placed on a laboratory mouse in a tight-fitting wire-mesh cage where the activity

of the mouse is restrained. After the chiggers have attached to the mouse for feeding, they (mouse and chiggers) are transferred to a loose-fitting cage [30]. Michener [32] also reported to use domestic chickens to be used as host of lab rearing chiggers. A tray (slightly bigger than the cage) filled with water and the cage are held above the surface of water and used to catch engorged larvae when they drop from the mouse [30]. For chiggers that do not parasitize mammals, snakes and turtles can be used to feed larvae [34]. The temperature for rearing *L. deliense* and *L. fletcheri* is 27 ± 3 °C [31]. The optimal relative humidity is 80–100% [14,21]. In addition, Audy and Nadchatram [35] reported method of rearing chigger larvae collected from the field in glass tube fitted with wet strip of paper. This method was for only taxonomic study purpose.

One challenge of starting a colony from the field is the potential for the engorged chiggers to be infected with human pathogens, especially if the chiggers were collected from known endemic areas of disease. Additionally, there are areas where the status of chigger pathogens have not been investigated nor the transmission potential. These unknown concerns can complicate starting a laboratory colony, since BSL level III containment that involves the use of vertebrate animals would be needed if human pathogens are involved or potentially involved as part of the rearing effort.

3. Systematics Status

3.1. Taxonomy

Three classification systems for chiggers have been suggested: (*i*) all chiggers are in the family Trombiculidae Ewing, 1929 with three subfamilies, Trombiculinae, Ewing, 1929, Leeuwenhoekiinae, Womersley, 1944 and Apoloniinae Wharton, 1947 [36–39]; (*ii*) all chiggers are in two families, Trombiculidae and Leeuwenhoekiidae Womersley, 1945 [5,17,40]; and (*iii*) all chiggers are in in three families, Trombiculidae, Leeuwenhoekiidae and Walchiidae [41–43]. There is disagreement on whether the taxa should be included in the family Trombiculidae [44]. In this review, we are using the classification that chiggers are the larval mites in the families Trombiculidae and Leeuwenhoekiidae.

Walters et al. [17] summarized all publications from 1923 to 2009 that reported chiggers in North America, the chigger species reported in each publication, the hosts that the chiggers were found, and the US state or Canadian province where they were associated. The list contained 42 species in Leeuwenhoekiidae and 205 species in Trombiculidae in the US [17]. In 2015, Crossley and Clement [45] reported a new species, *Hoffmanniella solickiana*, from the Rafinesque's Big-Eared Bat in Georgia, US, making a total of 248 chigger species from the US, containing 42 species in Leeuwenhoekiidae and 206 species in Trombiculidae. The chigger species richness by state in the US is diverse (Figure 4).

Chiggers are reported in every state except Hawaii in the United States. The states that reported more than 30 chigger species were California (97 species), Texas (74), Utah (52), Kansas (47), Colorado (30) and Nevada (30). The chigger species were more diverse in the Southwest. Some species in the genus *Leptotrombidium* were found in the US, i.e., *Leptotrombidium myotis*, *L. panamensis*, and *L. peromysci*. The genus *Leptotrombidium* is known as the vector of *O. tsutsugamushi*, the causative agent of scrub typhus.

Based on the list from Walters et al. [17], *E. alfreddugesi* (Oudemans 1910) was the most widely spread chigger in the US, found in 22 states from 179 host species, including reptiles, amphibians, birds and mammals. However, Bennett et al. [46] argued that the most common chigger in the eastern US, *E. alfreddugesi*, was possibly misidentified and should be replaced with *E. cinnabaris*. Loomis and Wrenn [47] investigated the systematics of the genus *Eutrombicula* and found that this genus in the Eastern US needed revision since some of the records were misidentified. This species was named by Oudemans as *Microthrombidium alfreddugesi* in 1910 based on four specimens collected from Temascaltepec, close to Tejupilco, Mexico, which was the type species for the genus *Eutrombicula*, Ewing, 1938 [48]. Fuller [49] revisited the two representative specimens of Oudemans' collection and found these two specimens were shrunken and not transparent enough for study. W. J. Wrenn argued that it was unlikely that the two improperly mounted specimens belonged

to the common *Eutrombicula* species in the Eastern US, where there is a large number of species in the genus *Eutrombicula* in the US [46,47]. Another synonym on Fuller's list for this species is *Trombicula cinnabaris*, Ewing, 1920 [49]. D. A. Crossley rechecked Ewing's specimens named *Trombicula cinnabaris* and found several morphological characteristics were in accordance with the illustrations of *E. alfreddugesi* [46]. Thus W. J. Wrenn concluded that *E. cinnabaris* was the most suitable name for the species that was formerly classified as *E. alfreddugesi* [46,47]. Wrenn continued to use *E. cinnabaris* for his publications [50,51]. In the world checklist [5] and the North American checklist [17] of Trombiculidae and Leeuwenhoekiidae, *E. alfreddugesi* and *E. cinnabaris* were listed as two species. The possible reason for this could be the new name for this species that was proposed, but others treated it as another species and still used the old name. It was suggested by Loomis and Wrenn [47] that the genus *Eutrombicula* needed revision, and this revision is greatly overdue.

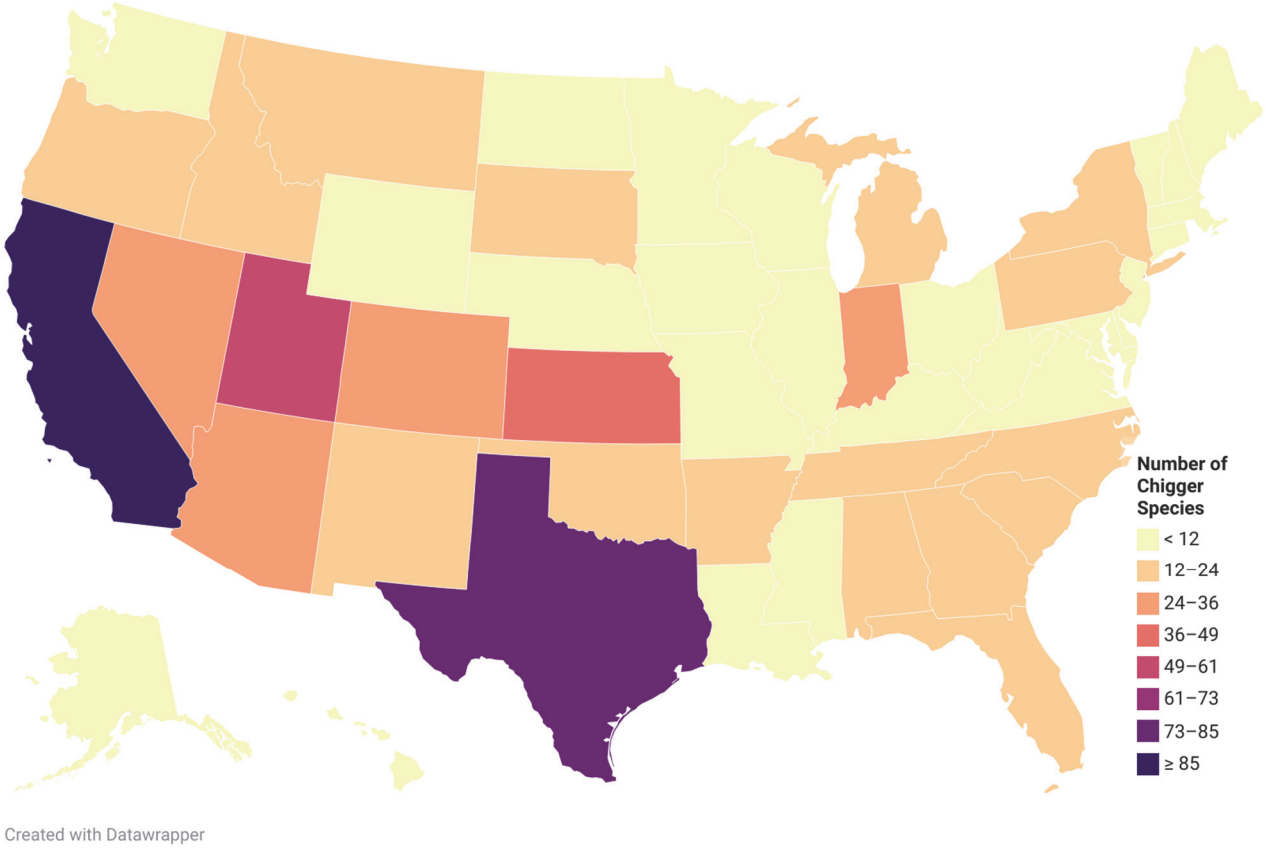


Figure 4. Chigger species richness in the US. Created with Datawrapper. Data sources: [17,45].

3.2. Identification

The taxonomy of chiggers almost exclusively relies on larval morphological characteristics [52–55]. A chigger's body is divided into two sections, the gnathosoma and idiosoma. The gnathosoma is the chigger mouthparts comprised of four parts: the cheliceral blades, palps, cheliceral base and gnathobase [36]. The shape of the scutum (usually the only sclerotized plate on the idiosoma, next to the gnathosoma), the number and arrangement of dorsal setae, chaetotaxy of legs, leg segmentation, and different forms or length of the gnathosoma (the cheliceral blades, the galeal setae, the palpal setae and the palpal claw), etc. are used for chigger identification.

Chiggers typically were cleared in lactophenol for them to be transparent and easy to observe under the microscope for classification [56–58]. After clearing, chiggers are then mounted dorsal-ventrally in mounting media like Hoyer's medium [59] or Berlese fluid [60] on glass slides and dried at 50 °C before identification under a high magnification microscope using differential interference contrast [61]. More recently methods to clear and

mount ectoparasites has improved, e.g., lactic acid, phenol and iodine and water are used for the preparation of rapid clearing solution for chiggers [62].

The identification relies on published dichotomous keys based on morphological characteristics. Some of the published keys are specific for a particular region. For example, Nadchatram and Dohany [63] provided keys for Southeast Asian chiggers to the subfamily, genus and subgenus; Brennan and Goff [64] provided a key to the western hemisphere (Nearctic and Neotropical regions) to the genus level; and Fernandes and Kulkarni [35] provided a key of Indian chiggers to the subfamily, tribe, genus and species. There is also separate guidance for species-level identification. For example, Vercammen-Grandjean and Langston [65] and Stekolnikov [66] provided keys to *Leptotrombidium* which can be used to identify *Leptotrombidium* chiggers into species.

Identifying chiggers using morphological characteristics is complicated and also time-consuming as chiggers are tiny and require days for sample preparation before identifying them under a high magnification microscope. Another challenge is to find a suitable key to guide the identification process, and sometimes no keys are available for certain species since the keys are not up to date. This identification largely relies on personal experiences. Researchers often need to send samples to experts to verify their identification [67,68]. Current knowledge of intraspecific variance is not sufficient, and some species are described from only a few specimens [46]. For measurements of body parts of chiggers, different researchers will likely have different results depending on how the measurements were made. In the future, chigger identification should consider not only morphological characteristics but also molecular tools to improve accuracy.

The molecular data for chigger identification are limited, especially in the US. The nucleotide data on chiggers in GenBank (Access date: 23 March 2022) was examined and summarized in Table 1. There were 10,465 entries in total and molecular data for 47 chigger species. Data for 36 species were published in a peer-reviewed journal while 11 species have not been published. Given the number of species of chiggers worldwide, the molecular data in GenBank is greatly limited, only making up 1.5% (47/3013) of the total number of species. This was surprising considering the significant health risks to humans from these mites. We categorized the published chigger data available in GenBank by country. The molecular data were mostly found in Southeast Asian and South American countries. The only species with molecular data available from the US was for *E. splendens* [69,70] with a complete sequence provided of the small and large subunit ribosomal RNA gene.

Table 1. Summary of chigger molecular data in GenBank in peer-reviewed publications.

Country (Continent)	Different Chigger Species in GenBank	% *	Peer Reviewed Publications	Chigger Species Reported	Target Genes *
Laos (Asia)	11	30.56	[71]	*Ascoschoengastia indica*	COI
				Blankaartia acuscutellaris	COI
				Leptotrombidium deliense	18S, COI, whole genome shotgun
				Microtrombicula chamlongi	COI
				Schoengastia kanhaensis	COI
				Walchia alpestris	COI
				Walchia ewingi ewingi	COI
				Walchia ewingi lupella	COI
				Walchia kritochaeta	COI
				Walchia micropelta	COI
				Walchia minuscuta	COI

Table 1. *Cont.*

Country (Continent)	Different Chigger Species in GenBank	% *	Peer Reviewed Publications	Chigger Species Reported	Target Genes *
Thailand (Asia)	1	2.78	[72]	*Leptotrombidium deliense*	Mitochondrial (mt) DNA complete genome
China (Asia)	1	2.78	[73]	*Neoschoengastia gallinarum*	small subunit rRNA, large subunit rRNA, 5.8S, internal transcribed spacer 2, large subunit rRNA, COI
Korea (Asia)	2	5.56	[74]	*Helenicula miyagawai* *Leptotrombidium scutellare*	18S mt DNA complete genome, COI, 18S, 5.8S
Japan (Asia)	1	2.78	[75]	*Leptotrombidium pallidum*	COI, mt DNA complete genome, 5.8S, whole genome shotgun
Malaysia (Asia)	1	2.78	[76]	*Leptotrombidium fletcheri*	mt DNA complete genome
Canada (North America)	1	2.78	[77]	*Neotrombicula microti*	COI
US (North America)	1	2.78	[69,70]	*Eutrombicula splendens*	large subunit rRNA, small subunit rRNA, 28S, 18S, *Ef1-α*, *Srp54*, *Hsp70*, COI
Mexico (North America)	1	2.78	[70]	*Acomatacarus arizonensis*	28S, 18S, COI, *Ef1-α*, *Srp54*, *Hsp70*
Brazil (South America)	12	32.33	[78,79]	*Herpetacarus*(*Arisocerus*) *hertigi* *Blankaartia sinnamaryi* *Eutrombicula daemoni* *Eutrombicula goeldii* *Eutrombicula tinami* *Fonsecia ewingi* *Hannemania hepatica* *Hannemania yungicola* *Quadraseta brasiliensis* *Quadraseta pazca* *Quadraseta trapezoides* *Trombewingia bakeri*	small subunit rRNA small subunit rRNA small subunit rRNA small subunit rRNA 18S 18S 18S small subunit rRNA, 18S small subunit rRNA small subunit rRNA small subunit rRNA
Poland (Europe)	3	8.33	[80,81]	*Hirsutiella zachvatkini* *Miyatrombicula muris* *Neotrombicula inopinata*	COI COI COI
Spain (Europe)	1	2.78	[82]	*Morelacarus* sp.	COI
Madagascar (Africa)	1	2.78	[83]	*Schoutedenichia microcebi*	small subunit rRNA

* %: Percent of different chigger species from each country out of the total 36 different species in GenBank in peer-reviewed publications. * Gene abbreviations: COI, cytochrome *c* oxidase I; 18S, 18S ribosomal RNA gene; 5.8S, 5.8S ribosomal RNA gene; *Ef1-α*, elongation factor 1alpha100E; *Srp54*, signal recognition particle protein 54k gene; *Hsp70*, Hsc70-5 heat shock protein cognate 5 gene.

We constructed a Neighbor-Joining tree of the small subunit rRNA sequences for chiggers collected from American countries (Figure 5). The chigger small subunit rRNA

sequences data were downloaded from GenBank only when the data were also published in peer-reviewed journals. *Eutrombicula tinami*, *Quadraseta trapezoides* and *Trombewingia bakeri* representing different genera, were clustered together in our analysis (Figure 5). *Herpetacarus hertigi*, *Blankaartia sinnamaryi*, and *E. daemoni* are more closely related to each other than they were *E. splendens* and *E. goeldi* in the tree. These results suggest that using the current available sequences in GenBank to identify chiggers would most likely lead to incorrect identifications.

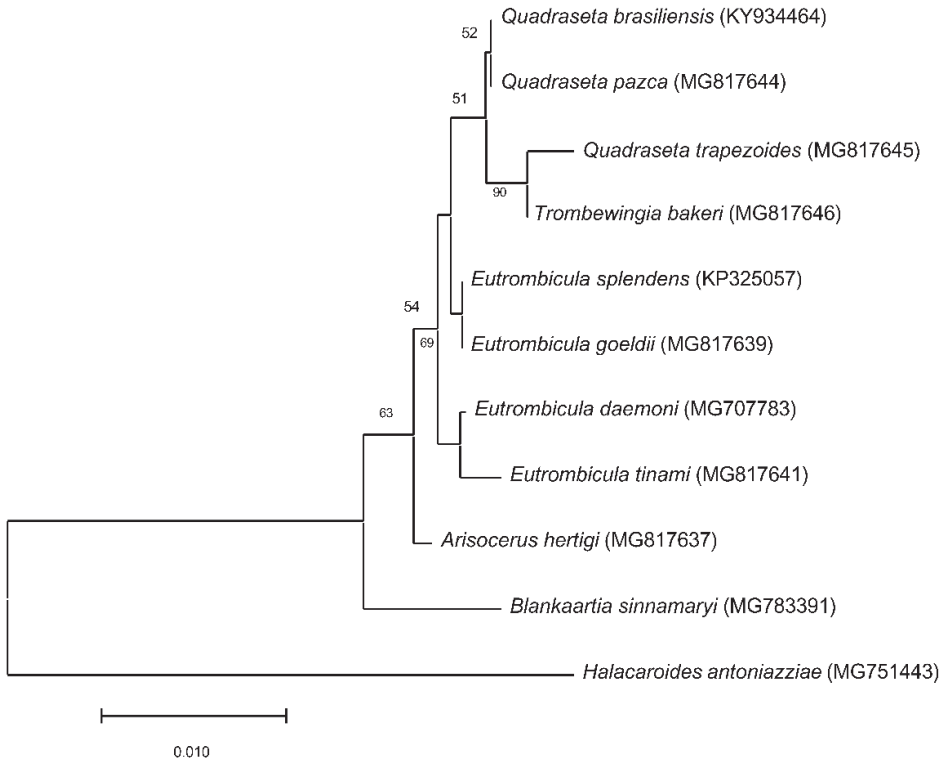


Figure 5. Neighbor-Joining tree of small subunit rRNA sequences of chiggers collected from North and South American countries. The chigger small subunit rRNA sequences data were downloaded from GenBank only from peer-reviewed journal publications. The *Halacaroides antoniazziae* (MG751443) was used as an outgroup. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown below the branches. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site. This analysis involved 10 nucleotide sequences. All positions containing gaps and missing data were eliminated (complete deletion option). There were a total of 403 positions in the final dataset. Evolutionary analyses were conducted in MEGA11.

Obtaining chigger molecular data for the US is long overdue. One of the reasons this is lacking is the difficulty in making a morphological identification and at the same time obtaining molecular data. The sample preparation process for chiggers requires the use of lactophenol or acidic phenol for clearing and heat drying (discussed earlier). This process destroys the DNA. There are two solutions available for researchers to resolve this issue. Lee et al. [84] modified the method from Dohany et al. [85] and Ree et al. [86] for the detection of *O. tsutsugamushi* from chiggers using a fluorescent antibody test. Lee et al. [84] used a fine needle to puncture the dorsal-posterior part of the chigger and squeezed out internal tissue for DNA extraction while the exoskeleton was retained mostly intact for morphological identification. Kumlert et al. [71] argued that this method affects the morphological identification, and the DNA extraction process is challenging because of

the limited amount of tissue obtained by this method. Kumlert et al. [71] alternatively used the autofluorescent characteristics of the chigger exoskeleton and developed an identification method using a combination of autofluorescent and brightfield microscopy imaging. Chiggers were mounted in sterile water or ethanol to prevent DNA damaged by clearing agents and saved for molecular identification later.

Having a database containing the sequences of mitochondrial cytochrome oxidase subunit I gene (*COI*) and nuclear genome fragments at a minimum is essential to building a reliable molecular identification method for chiggers. A substantial systematics investment is needed to achieve this objective for chiggers in the US. There is also an increasing interest of using high resolution, three channel imaging coupled with artificial intelligence using multi-layered neural networks to classify closely related species of almost identical-looking moth eggs (Roe, in review) that could be applied to chigger classification. This approach is especially useful for classifying small objects and because the system is automated, has the potential for high throughput.

Chiggers are a poorly understood group of arthropods with a significant importance to human health that *in toto*, are understudied at many levels including systematics and classification. Molecular tools not only could be used to identify the chiggers to species, but also be applicable for classification of the free-living deutonymph, adults and eggs.

4. Chigger Microbiome

Ponnusamy et al. [87] extracted DNA from *Orientia tsutsugamushi* infected and uninfected *Leptotrombidium imphalum* laboratory colonies at different life stages: larvae (chiggers), deutonymph, male adults and female adults from Thailand. The 16S rRNA V3-V4 region amplicons were amplified from the extracted DNA and sequenced using Illumina MiSeq. They reported *Orientia*, *Ralstonia* and *Propionibacterium* were the only three taxa that represented >5% of the relative bacteria abundance for *O. tsutsugamushi* infected chiggers. For *O. tsutsugamushi* uninfected chiggers, the *Ralstonia*, *Propionibacterium*, *Streptococcus* and *Corynebacterium* genera represented >5% the bacteria relative abundance [87]. Notably, unidentified taxa of Amoebophilaceae were found predominantly in *O. tsutsugamushi* infected female *L. imphalum* mites but with low relative abundance in larvae (chiggers) and male adult mites. This suggested a mutualistic relationship between Amoebophilaceae and *O. tsutsugamushi* in *L. imphalum* mites that needs further study [87].

Alghamdi [68] extracted DNA from ten species of chiggers collected from rodents in southwestern Saudi Arabia: *Ascoschoengastia browni*, *Ericotrombidium caucasicum*, *Ericotrombidium kazeruni*, *Microtrombicula microscuta*, *Pentidionis agamae Schoutedenichia saudi*, *Gahrliepia lawrencei*, *Schoutedenichia thracica*, *Schoutedenichia zarudnyi* and *Helenicula lukshumiae*. Chigger species were identified using an autofluorescence method [71]. Alghamdi amplified the 16S rRNA V3-V4 region from the extracted DNA and sequenced the 16S rRNA libraries using Illumina. *Corynebacterium*, *Mycobacterium*, *Staphylococcus*, *Candidatus Cardinium*, *Burkholderiaceae* and *Wolbachia* were found to be dominant in all the chigger samples. Especially, *Wolbachia* had a high relative abundance in *P. agamae*. Alghamdi [68] reported that *Orientia* OTUs were observed from *E. kazeruni* and *P. agamae* with a low relative abundance and had 98.88% similarity with the *Orientia chuto* sequence in NCBI. Alghamdi suggested that scrub typhus might exist in Saudi Arabia. More work is needed to confirm the *Orientia* sequence in chiggers.

Chaisiri et al. [88] studied the microbiome of nine chigger species that were collected on small mammals from 11 provinces in Thailand: *Ascoshoengastia indica*, *Blankaartia acuscutellaris*, *Helenicula kohlsi*, *Helenicula pilosa*, *Leptotrombidium deliense*, *Schoengastiella ligula*, *Walchia micropelta*, *Walchia minuscuta*, and *Walchia kritochaeta*. The chiggers were identified by autoflorescent imaging [71]. The 16S rRNA gene V4 region was amplified by PCR and sequenced by Illumina MiSeq. They reported that the dominant taxa across all the chigger samples were *Sphingobium*, *Mycobacterium*, Neisseriaceae and Bacillales. *Geobacillus* was found to be a potential arthropod endosymbiont. *Cardinium, Pseudonocardia and Rickettsiella* were presented in 20–45% of the pooled chigger samples while *Wolbachia* was detected in

3.18% of the pooled samples. *O. tsutsugamushi* and *Borrelia* spp. were detected in *L. deliense*. Potential human pathogens, *Staphylococcus* and *Haemophilus parainfluenzae*, were found in several chigger species.

The microbiome of chiggers from a Thailand lab colony and field samples from Thailand and Saudi Arabia was highly diverse. Not surprisingly, *O. tsutsugamushi* was detected in the Thailand chiggers. However, the finding of *Borrelia* spp. in chiggers [88] suggested they could be the vector for Borreliosis (Lyme disease), currently believed to be transmitted only by ticks. Chiggers might also harbor other human and animal pathogens like *Staphylococcus*. More work is needed to characterize the microbiome of different chigger species from different habitats to better understand if bacteria will affect the vector competence of chiggers, and to better understand if there are any other pathogens that chiggers can vector other than *Orientia.*

5. Do Chiggers Transmit *Rickettsia* and *Orientia* in the US?

5.1. Summary of Zoonotic Bacteria and Viruses Transmitted by Chiggers

Leptotrombidium spp. are vectors of *Orientia tsutsugamushi* that cause Scrub typhus, a life-threatening disease with up to a 50% fatality rate if left untreated [89]. There are 46 chigger species reported to be infected with *O. tsutsugamushi* [90]. Eleven *Leptotrombidium* species were proven to be vectors of scrub typhus [12]. *Leptotrombidium* spp. are both the vector and reservoir of *O. tsutsugamushi* [91]. The high transovarial and transstadial transmission rates among chigger species play an important role in maintaining the disease in nature, since chiggers only feed on an animal host once [92]. Whether rodents also serve as the reservoir for *O. tsutsugamushi* needs further investigation; rodents could only be dead-end hosts [93].

Scrub typhus was originally endemic in the tsutsugamushi triangle. This traditional triangle area extends north to Japan and Russia, south to Northern Australia, and west to Afghanistan and Pakistan [94]. Recent studies suggest scrub typhus is not limited to this triangle. Scrub typhus cases were reported in the United Arab Emirates, and a new *Orientia* species *O. chuto* was isolated from a patient in 2006 [95]. Scrub typhus cases reported in Chile [96,97] and serologic evidence in Peru [98] suggested scrub typhus is endemic in South America. Furthermore, *Orientia* sequences were detected in rodents from France [99], Senegal [99] and Kenya [100]. Besides some *Leptotrombidium* species, *Schoengastiella ligula* has been implicated as a vector of scrub typhus in India [101]. Thus, a larger geographic distribution of scrub typhus, potential new *Orientia* species and new vector chigger species should be taken into account. The potential of *Orientia* species establishing in the US is high given the number of chigger species found throughout the US but has never been seriously studied [17].

Chiggers are also reported to play a role in Hantavirus transmission, a disease previously thought to be transmitted through aerosol exposure to rodent saliva, urine and feces (CDC https://www.cdc.gov/hantavirus/, accessed on 19 May 2022). Hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS) are the two clinical syndromes caused by hantavirus when transmitted from rodents to humans [102]. In the United States, hantavirus cause hantavirus pulmonary syndrome (HPS) instead of HFRS, and HPS is a severe and sometimes life-threatening respiratory disease [103]. Wu et al. [104] studied the role of *L. scutellare* in the transmission of hantavirus that cause HFRS in China. They reported hantavirus were isolated from (*i*) rats trapped in the HFRS endemic areas of Shaanxi Province, China and also from chiggers on the rats; (*ii*) from eight previously hantavirus negative mice placed in metal cages in the field to lure chiggers; (*iii*) from mice bitten by virus-positive chiggers; and (*iv*) from larvae hatched from eggs collected from endemic areas. Wu et al. [104] concluded that *L. scutellare* could be the vector of hantavirus and able to transmit the virus to its offspring and rodent hosts by biting. A review of the recent studies of mites and hantavirus in China also indicated that the hantavirus can be maintained in chiggers by transovarial transmission [105]. Rodent ectoparasite screening in Texas detected two chiggers collected from these rodents and one free-living chigger

collected from the soil that were positive for hantavirus specific RNA [106]. Although cumulative cases in the US have occurred predominately in the western states, only 10 states have been immune to its presence (https://www.cdc.gov/hantavirus/surveillance/index.html, accessed on 19 May 2022). The possible role of chiggers in the transmission of hantavirus in the US needs further investigation.

Bartonella tamiae was isolated for the first time from patients to determine the etiology of three febrile cases in Thailand [107]. In another study, chiggers in the genus *Leptotrombidium*, *Schoengastia*, and *Blankarrtia* were collected from rodents in Thailand, and the 16S–23S intergenic spacer region and citrate synthase gene (*gltA*) region were targeted by PCR to screen for *B. tamiae* DNA in these chiggers [108]. In this work, 29 of the 40 chigger samples tested positive for *B. tamiae* DNA, and there were 97.7–100% sequence homology between the *Bartonella* DNA detected in this study and the *Bartonella* species isolated from three patients in Thailand. This suggested that chiggers play a potential role in the transmission of *Bartonella* to humans.

Rickettsia species have also been found in chiggers. *Rickettsia* spp.TwKM02 and TwKM03 were detected in *Leptotrombidium* chiggers from rodents in two islets in Taiwan; the two *Rickettsia* species were close to *R. australis* and *R. felis* URRWXCal2 based on a phylogenetic tree constructed on partial *gltA* gene sequences [109]. *R. australis*, *R. japonica*, *R. felis*, *R. typhi*, *R. akari*, *Rickettsia* sp. TwKM02, *R. conorii* and *Rickettsia* sp. Cf15 were detected in chiggers collected from rodents in South Korea [110]. A new *Rickettsia* species found in *L. scutellare* from rodents in Shandong, China was close to *R. australis* and *R. akari* [111]. *Rickettsia* spp. were detected in chiggers, *Herpetacarus hertigi*, *Quadraseta trapezoides* and *Trombewingia bakeri*, collected from rodents in Brazil and was identical to "*Candidatus* Rickettsia colombianensi" based on the amplified *gltA* and *ompA* genes [79]. *Rickettsia* spp. found from soil-inhabiting unfed *L. scutellare* chiggers in Japan were close to *R. akari*, *R. aeschlimannii*, *R. felis* and *R. australis* [112]. Since chiggers in this study were unfed, this suggested *Rickettsia* spp. were transstadially and/or transovarially transmitted in chigger mites [112].

Except for *O. tsutsugamushi* that cause scrub typhus, chiggers mostly are not considered as vectors for rickettsioses. Given that some *Rickettsia* spp. found in chiggers were similar to species in the spotted fever group (SFG), e.g., *R. aeschlimannii*, *R. felis* and *R. australis* (recognized because of their infectivity and pathogenicity as *Rickettsia* spp.), the possible role chiggers might play in the transmission of *Rickettsia* to humans deserves further investigation. A well, a pool of chiggers with *Rickettsia* spp. TwKm02 were positive for *O. tsutsugamushi* in Taiwan [109]. Therefore, the possibility of coinfection of SFGR and scrub typhus in chiggers needs further study. Additionally, *R. felis* and other *Rickettsia species* were detected in chiggers collected from rodent in the US [23]. It is possible that rickettsioses in the US attributed to ticks might also originate from chiggers. Since the chigger microbiomes in the US have been minimally studied, this remains a possibility.

Borrelia species have also been found in chiggers. *Borrelia burgdorferi* sensu lato (s.l.) was found in 634 *Neotrombicula autumnalis* chiggers collected on 100 wild-caught rodents along with 1380 questing chiggers on vegetation, using PCR and DNA hybridization in Germany and *Borrelia* DNA was detected from a chigger collected from a greater white-tooted shrew [113]. An infection experiment also was conducted by Kampen et al. [113] by placing 305 questing unfed chiggers on *B. garinii* infected laboratory mice and on *B. afzelii* infected laboratory Mongolian gerbils. *Borrelia* DNA was detected on a pool of four chiggers that fed on *B. garinii* infected laboratory mice, and a nymph kept from a larva that was previously fed on *B. afzelii* infected laboratory Mongolian gerbils. Results suggested a possible transmission of *Borrelia* spp. from *N. autumnalis* to mice and gerbils. *Borrelia* spp. DNA was also screened on 509 chiggers in the genus *Neotrombicula* that were collected from 1080 wild birds in Certak, Czech Republic [114]. One sample pooled with five *Neotrombicula* chiggers removed from one Eurasian blackcap (*Sylvia atricapilla*) tested positive for *B. burgdorferi* s.l. by PCR and a reverse line blotting assay [114].

5.2. Role of Endosymbionts in Orientia Infection

Takahashi et al. [115] reported that the adults in *O. tsutsugamushi* infected *Leptotrombidium fletcheri*, *L. arenicola* and *L. deliense* colonies were almost entirely made up of females, and this female-biased sex ratio represents a feminization. After treating the *O. tsutsugamushi* infected *L. fletcheri* colonies with antibiotics, a high ratio of adult males occurred. Takahashi et al. [115] suggested *O. tsutsugamushi* inhibits the development of males. Recent microbiome studies detected *Wolbachia* and *Cardinum* sequences in chiggers [68,88]. *Wolbachia* and *Cardinum* are facultative bacterial endosymbionts of arthropods, commonly maternally inherited and associated with reproductive manipulation such as feminization and parthenogenesis [116]. It is possible that *Wolbachia* and *Cardinum* can also cause feminization in some chigger species infected with *O. tsutsugamushi.*

Feminization also occurred in *O. tsutsugamushi* infected F1 generation adults of *L. imphalum* [117]. A microbiome study of a *L. imphalum* laboratory colony found an unidentified species, Amoebophilaceae, which dominated in *O. tsutsugamushi* infected female adults and was at a very low relative abundance in *O. tsutsugamushi* uninfected mites [87]. The results suggested a mutualistic relationship between *O. tsutsugamushi* and the unidentified species in the Amoebophilaceae [87]. It is possible that this unidentified species in Amoebophilaceae was responsible for the feminization of *L. imphalum* adults. Further characterization and pathogenicity of this Amoebophilaceae species is needed including understanding their possible contributing role in pathogenesis with other bacteria.

6. Possible Misdiagnosis of Tick-Borne Diseases and Allergies

Tick-borne diseases in the US include spotted fever rickettsioses, Lyme disease, ehrlichiosis, anaplasmosis, tularemia, babesiosis, Colorado tick fever, and relapsing fever [118]. Until recently, most patients with tick-associated rickettsiosis were considered to have Rocky Mountain spotted fever (RMSF), caused by *R. rickettsii*. Serological techniques like indirect the immunofluorescence assay (IFA) was commonly used for the routine diagnosis of RMSF [119,120]. However, other *rickettsia* species in the Spotted Fever Group (SFG) can also elicit antibodies that are cross-reactive with *R. rickettsia* (serological cross-reactions) [118]. Additionally, other SFG rickettsia species, including *R. parkeri* and *Rickettsia* species 364D [121] can cause illness that was diagnosed as RMSF. SFG *rickettsia* species have been detected in chiggers [79,109–112,122], suggesting that reported tick-associated rickettsiosis may have some level of chigger associated.

Borrelia spp. DNA was detected from *Neotrombicula autumnalis* collected from wild birds in the Czech Republic [114]. Additionally, a recent microbiome study of *L. deliense* collected from rodents in Thailand showed that *Borrelia* spp. were found in 49.2% of the chigger samples [88]. Larval ticks, sometimes known as "seed ticks," bite humans and are nearly impossible for non-professionals to distinguish from chigger bites. In a sero-epidemiological study conducted on France forestry workers, a large number of people who were seropositive to *B. burgdorferi* were unable to recall a tick bite [123]. The reasons could be those workers were bitten by larval ticks or chiggers.

It is now well recognized that the carbohydrate galactose-α-1,3-galactose (alpha-gal) which is present in some tick species can elicit an alpha-gal specific IgE immune response [124]. People who were reported to be bitten by ticks have developed a hypersensitivity to red meats, including beef and lamb, called red meat allergy [125]. The symptoms of red meat allergy range from mild to severe, some can be acute and fatal [124]. Notably, people in three cases who had severe red meat allergy later reported having pruritic bites from chiggers either on their lower legs or at the waistband area [126]. It is possible that chiggers could also contribute to the prevalence of red meat allergy. The searching for alpha-gal antigens in chiggers should be investigated to determine if chiggers can also cause red meat allergy.

7. Chigger Control

Chiggers are frequently difficult to control chemically [127]. For successful management, large areas would have to be sprayed numerous times [127]. The best way to prevent chigger bites is to avoid them [128]. The methods of avoiding chigger bites are summarized in Table 2.

Table 2. Methods of avoiding chigger [127–129].

Scenarios	Methods of Avoiding Chigger Bites
When venturing into chigger habitat	Wear permethrin-treated clothing Apply repellents like DEET Wear tightly woven long sleeves, long pants, and closed-toe shoes Tuck shirts into pants, tuck pants into socks Stay in the middle of the trail, avoid high bushes and tall grass Avoid sitting directly on the ground
After you come inside	Remove clothing immediately and launder it in hot water Wipe down shoes and boots Take a hot and soapy shower with a vigorous skin massage
Yard	Try to keep small mammals out of the yard by putting up a fence and keeping trash can lids secured keep turf short and vegetation trimmed

When venturing into chigger habitat, wearing permethrin-treated clothing and applying repellents containing DEET (diethyltoluamide) is effective against chiggers [129]. Wearing tightly woven long sleeves, long pants, closed-toe shoes and tucking shirts into pants and tucking pants into socks can keep chiggers on the outside of your clothing. There are also non-insecticidal garments available commercially like Rynoskin Total that claim to protect from chiggers, mainly based on preventing access under the garments.

When outdoors, stay in the middle of trails and avoid high bushes and tall grass that might have chiggers. It is also suggested to avoid sitting directly on the ground to reduce chigger encounters [130]. Upon return, it is recommended to remove clothing and wash clothing immediately. It is also helpful to wipe down shoes and boots to avoid bringing chiggers into the home. Taking a hot and soapy shower with a vigorous skin massage will help remove chiggers attached to your body [128].

To reduce chigger populations in the yard, keep turf grass short and vegetation trimmed, since high temperature and low humidity conditions are inhospitable for chiggers. Another approach is to keep small mammals out of the yard using fencing and keeping trash can lids secured to make the trash less attractive [129].

8. Conclusions

Overall, chiggers have been understudied world-wide but especially so for the US. The published research mainly focuses on taxonomy (identification methods), ecology, and reports of the discovery of chiggers on various hosts in the US. However, the life history of other stages other than larvae needs further study.

There are 248 chigger species reported from the U.S. to date. Chiggers are reported in every state except Hawaii in the United States. The taxonomy of chiggers almost exclusively relies on larval morphological characteristics; molecular data on chiggers are limited. It is likely that the most common chigger in North America, *E. alfreddugesi*, has been misidentified and should be replaced with *E. cinnabaris*. Building a reliable molecular identification method for chiggers correlated with morphological explicit characters is greatly needed. The molecular data are needed to better identify and study not only the parasitic stage but also free-living deutonymphs and adults correlated to what we already know about the larval stage. Three microbiome studies of chiggers were conducted from Thailand *L. imphalum* lab colonies, ten chigger species collected from rodents in Saudi Arabia, and nine chigger species collected from small mammals in Thailand. More work is

needed in this area to characterize the microbiome of different chigger species from different habitats to better understand if bacteria will affect the vector competence of chiggers, and to better understand if pathogens in addition to *Orientia* are transmitted by these mites. Thus far, data suggests pathogen competence in chiggers for scrub typhus might be associated with co-infection with other bacteria.

Scrub typhus was reported outside of the Tsutsugamushi Triangle. In addition to *Leptotrombidium* species, new chigger species were reported to vector the bacteria that causes scrub typhus. The potential of *Orientia* species establishing in the US is high given the number of species found throughout the US but has been minimally investigated. Chiggers are reported to harbor Hantavirus, *Bartonella*, *Rickettsia* and *Borrelia* suggesting a need for further study in North America. The role that chiggers play in disease transmission dynamics in the US is essentially unknown. It is possible some of the tick-borne diseases and red meat allergies are caused by chiggers.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijerph192215147/s1, Table S1: Search strategy used for Figure 1 was #1 AND #2 from Web of Science Core Collection; Table S2: Summary of publications of chiggers in the US. Publications were searched through the Web of Science on 3 May 2022; Table S3: Number of chigger species reported in each US states. References [5,16,17,20,22,26,34,40,45,46,48,52–58,64,67,106,131–193] are cited in the Supplementary Materials.

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Article

Mechanical Acaricides Active against the Blacklegged Tick, *Ixodes scapularis*

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Simple Summary: *Ixodes scapularis*, also known as the blacklegged tick or deer tick, is the vector of the bacteria that causes Lyme disease in humans, the most common vector-borne disease in the United States. Synthetic chemical pesticides are used to control ticks. Environmentally friendly, new methods are needed to manage chemical pesticide resistance. We evaluated the efficacy of the industrial mineral, Celite 610, an amorphous silica, against unfed *I. scapularis* nymphs. Celite is found in nature and has a mechanical, non-toxic mode of action. Dipping ticks into Celite for 1–2 s resulted in 90% mortality in as little as 69 min. Scanning electron microscopy suggested that one mode of action could be the physical obstruction of respiration. We developed another industrial mineral made from volcanic glass, Imergard^{TM} WP, for mosquito and filth fly control. In studies here, Imergard had similar activity as Celite against the deer tick. This research, although needing further study, suggests that industrial minerals could be a new, safe ("found in toothpaste") and persistent ("it's rock") alternative to chemical pesticides to control ticks.

Abstract: Cases of Lyme disease in humans are on the rise in the United States and Canada. The vector of the bacteria that causes this disease is the blacklegged tick, *Ixodes scapularis*. Current control methods for *I. scapularis* mainly involve chemical acaricides. Unfortunately, ticks are developing resistance to these chemicals, and more and more, the public prefers non-toxic alternatives to chemical pesticides. We discovered that volcanic glass, Imergard^{TM} WP, and other industrial minerals such as Celite 610 were efficacious mechanical insecticides against mosquitoes, filth flies, and agricultural pests. In this report, when 6–10- and 50–70-day old unfed *I. scapularis* nymphs were dipped for 1–2 s into Celite, the time to 50% mortality (LT_{50}) was 66.8 and 81.7 min, respectively, at 30 °C and 50% relative humidity (RH). The LT_{50} was actually shorter at a higher 70% RH, 43.8 min. Scanning electron microscopy showed that the ticks were coated over most of their body surface, including partial to almost total coverage of the opening to their respiratory system. The other mechanical insecticide, Imergard, had similar efficacy against blacklegged unfed nymphs with an LT_{50} at 30 °C and 50% RH of 70.4 min. Although more research is needed, this study suggests that industrial minerals could be used as an alternative to chemical pesticides to control ticks and Lyme disease.

Keywords: industrial minerals; Celite 610; Imergard^{TM} WP; diatomaceous earth; perlite

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1. Introduction

As the planet experiences a changing climate, incidences of arthropod vector-borne disease are on the rise as the habitat of insects and ticks expands. Tick-borne diseases make up 95% of vector-borne diseases in the United States and about 70% of these cases are Lyme disease; this makes Lyme disease the most common vector-borne disease in the US [1–3]. Lyme disease is caused by the spirochete bacteria, *Borrelia burgdorferi*, which causes serious illness that affects humans, companion animals, and livestock. This disease is transmitted by the bite of the blacklegged tick, *Ixodes scapularis*, and is a growing threat to public health throughout the US and Canada. *Ixodes scapularis* is responsible for the transmission of other

human pathogens including anaplasmosis, babesiosis, ehrlichiosis, and Powassan virus disease. The tick has an expanding geographic range, most likely due to climatic change, increasing the risk of human exposure to Lyme disease [4,5].

Controlling *I. scapularis* populations and preventing the public from being bitten by ticks is crucial to reducing Lyme disease and other tick-borne illnesses. There are numerous methods for preventing blood feeding on humans, i.e., repellents, permethrin-treated clothing, synthetic chemical pesticides to reduce wild tick populations on and off animals, reducing reservoir populations such as deer and rodents, and the vaccination of these reservoir hosts [1,6–8]. However, the majority of current tick control tactics rely heavily on synthetic chemical acaricides with few available and efficacious acaricide options derived from nature. Because the most common pesticides for vector control are used in agriculture, urban pest management, and tick control, the evolution of pest resistance to chemistry is increasing [9–12]. Numerous tick species have demonstrated resistance to chemical acaricides [13]. While this has not been reported yet in *I. scapularis*, it is possible this could occur in the future [13,14]. There is a need for environmentally friendly alternatives to permethrin (commonly used against ticks), which has been shown to have detrimental effects on non-target organisms [15]. Moreover, there is a large public demand for sustainable, safe, biorational alternatives to chemical pesticides.

We recently discovered two novel mineral-based insecticides for use in vector control, Celite 610 and Imergard™ WP. Celite is derived from a unique species of diatomaceous earth, and Imergard is 100% expanded perlite, an alumina–silica volcanic rock. Both Celite and Imergard work as mechanical insecticides, with Imergard being effective against mosquitoes and filth flies [16–18] and Celite against filth flies and sand flies (Roe, unpublished) and for the control of thrips in cotton [19]. Celite, under the trade name Deadzone, was also active against *A. americanum* [20].

The mode of action of mechanical insecticides is thought to be physical, through the abrasion of the cuticle and/or absorption of cuticular lipids, resulting in increased water loss and desiccation [21,22]. Mechanical insecticides have been shown to be effective on a variety of different types of insects such as weevils, grain beetles, whiteflies, bedbugs, cockroaches, filth flies, fleas, mosquitoes, and ticks [16,17,20,23–26]. Both perlite and diatomaceous earth-based products are non-toxic to vertebrates and occur naturally [16,20,27]. Perlite, the active ingredient in Imergard, was classified by the US Food and Drug Administration (FDA) as safe for use in juice filter aids and whitening agents in toothpaste [28,29]. Diatomaceous earth-based products such as Celite and Imergard can be stored indefinitely, are persistent, and because of their non-systemic mode of action, should not elicit cross-resistance to chemistry [20,21,30]. This study examines the efficacy and mode of action of Celite and Imergard on *I. scapularis*, the most important arthropod vector threatening human health in the US. Compared to the other life stages, the nymph was responsible for most Lyme disease cases [31].

2. Materials and Methods

2.1. Ticks and Industrial Minerals

Unfed *I. scapularis* (Acari: Ixodidae) nymphs were obtained from the Oklahoma State University tick laboratory (Stillwater, OK, USA). Once received, ticks were kept at least overnight in an insect rearing room at 27 °C, 70% relative humidity, and a 14:10 h light: dark cycle to acclimate and were used within 7 d. The ticks, at the time they were received, were of two different ages: 6–10 and 50–70 d post-ecdysis to the nymphal stage. The mechanical insecticides used in these assays were Celite 610 and Imergard™ WP, which were provided by the company Imerys (Imerys Filtaration Minerals, Inc., Roswell, GA, USA). The minerals were stored at room temperature in the dark in their original packaging containers until used.

2.2. Determining the Time to Mortality of I. scapularis after Exposure to Celite 610

Nymphs were exposed to Celite at either 6–10 d at 50% relative humidity (RH), 50–70 d at 50% RH, or 50–70 d at a high humidity of 70% RH. All assays were run at 30 ± 1 °C. Each tick was dipped for 1–2 s using a round #4 camelhair paintbrush (Craft Smart, Irving, TX, USA) into a plastic Petri dish bottom containing about 25 mg of Celite in an area of about 10 square mm. Ticks were submerged, exposing all surfaces to the Celite. After each dipping, ticks were moved to a clean plastic Petri dish bottom (5 ticks per plate (Fisherbrand 60 mm × 15 mm; Fisher Scientific, Hampton, NH, USA)), and the top of the plate was used to cover the bottom plate. The two plate halves were sealed together with Parafilm (Bemis Company, Inc., Neenah, WI, USA) to prevent ticks from escaping, and the Petri dish was incubated at 30 ± 1 °C, the respective assigned RH, and a 16L:8D photoperiod. Temperature and RH were measured with a Fluke 971 (Fluke Corporation, Everett, WA, USA) temperature and humidity meter. Ticks were monitored for death through the clear Petri dish top every 10 min until 100% mortality was obtained. Ticks were considered dead if they did not move after being disturbed by gently tapping the Petri dish. Controls followed the same protocol and were handled in the same manner, except that they were dipped into an empty Petri dish. Following the assay, the Petri dishes containing ticks were stored at −40 °C. After at least 48 h, ticks were individually placed in Eppendorf tubes (Fisherbrand 1.5 mL, Fisher Scientific, Hampton, NH, USA) and stored at −40 °C. The assays with 6–10 d old ticks at 50% RH were replicated three times with each replicate containing 10–15 ticks for a total of 40 ticks used in the treatment and 40 ticks used in the control. The assay with 50–70 d old ticks at 50% RH was replicated three times with each replicate containing 15–16 ticks, for a total of 46 ticks tested and 45 ticks used in the control. The high humidity (70% RH) assays used 50–70 d old ticks and were replicated three times, with each replicate containing 10–21 ticks for a total of 46 ticks for the treated assays and 45 for the controls. Each replicate consisted of 2–4 treatment and control Petri dishes, which were completed on different days. Ticks were examined at 24 h to confirm death.

2.3. Determining the Time to Mortality of I. scapularis after Exposure to Imergard™ WP

Unfed nymphs were 50–70 d old, and exposure followed the same methodology as the Celite assays, except we used Imergard. After dipping, ticks were incubated at 30 ± 1 °C, 50 ± 5% RH, and a 16L:8D photoperiod. Mortality was assessed every 10 min, as described earlier. Upon reaching 100% mortality, ticks were kept in the incubator and examined at 24 h to confirm death. This assay used a total of 40 treated ticks and 40 control ticks with 5 ticks in each Petri dish.

2.4. Statistical Analysis

Time course data were analyzed using a Probit model using the IBM® SPSS® Statistics 2021 software package, version 28 (Armonk, NY, USA). The Probit model was used to calculate the lethal time to achieve 50% mortality (LT_{50}), the lethal time to achieve 90% mortality (LT_{90}), the 95% confidence intervals (CI), and the $\chi 2$ of the time course data. There was no control mortality, and therefore Abbot's correction was not required. The data were normally distributed and did not require any transformation.

2.5. Scanning Electron Microscopy

Ticks that died by dipping into Celite and were stored without contact with each other at −40 °C were selected at random and examined by scanning electron microscopy (SEM). The selected ticks' SEM imaging was performed at the Analytical Instrumentation Facility at North Carolina State University (NCSU). Ticks were vacuum desiccated for 48 h and then mounted with super glue onto an aluminum Hitachi SEM mount. This was followed by Cressington sputter coating for 60 s with a 70 nm gold–palladium mixture (60 Au/40 Pd). The ticks were scanned with a Hitachi SU3900 (Hitachi, Ltd., Chiyoda City, Tokyo, Japan) variable pressure scanning electron microscope to image the Celite on the tick surface.

3. Results

3.1. Efficacy of Celite 610 against I. scapularis

The structure of Celite is shown in Figure 1. Mortality was first observed after 20 min for 6–12 d old ticks and after 30 min for 50–70 d old ticks at 50% RH with 100% mortality obtained at 140 and 170 min, respectively (Figure 2). At 70% RH, the first mortality for 50–70 d old ticks was at 20 min and 100% mortality at 100 min.

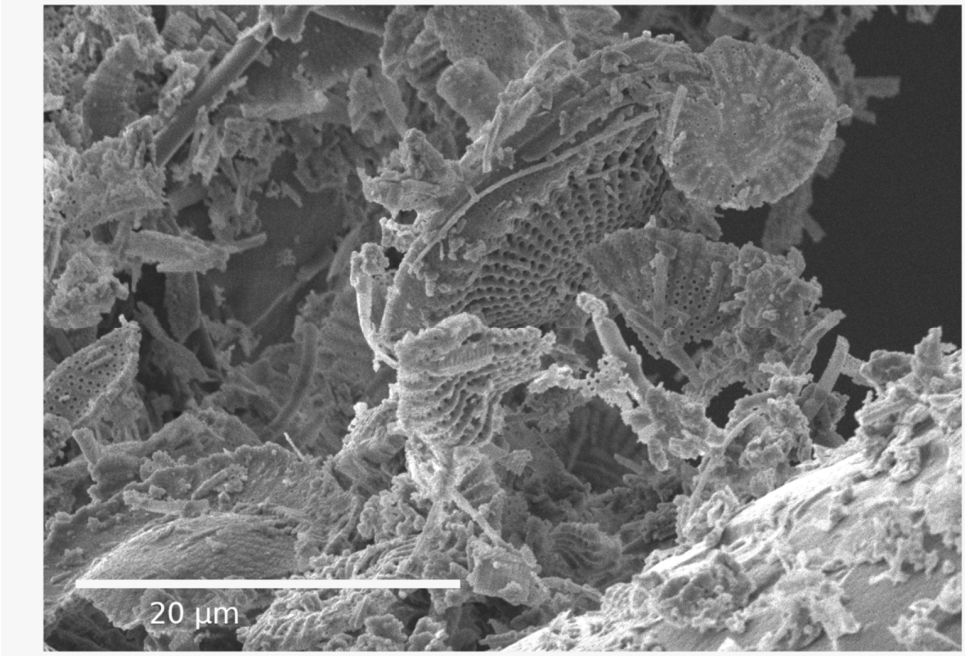


Figure 1. Scanning electron microscopy of Celite 610.

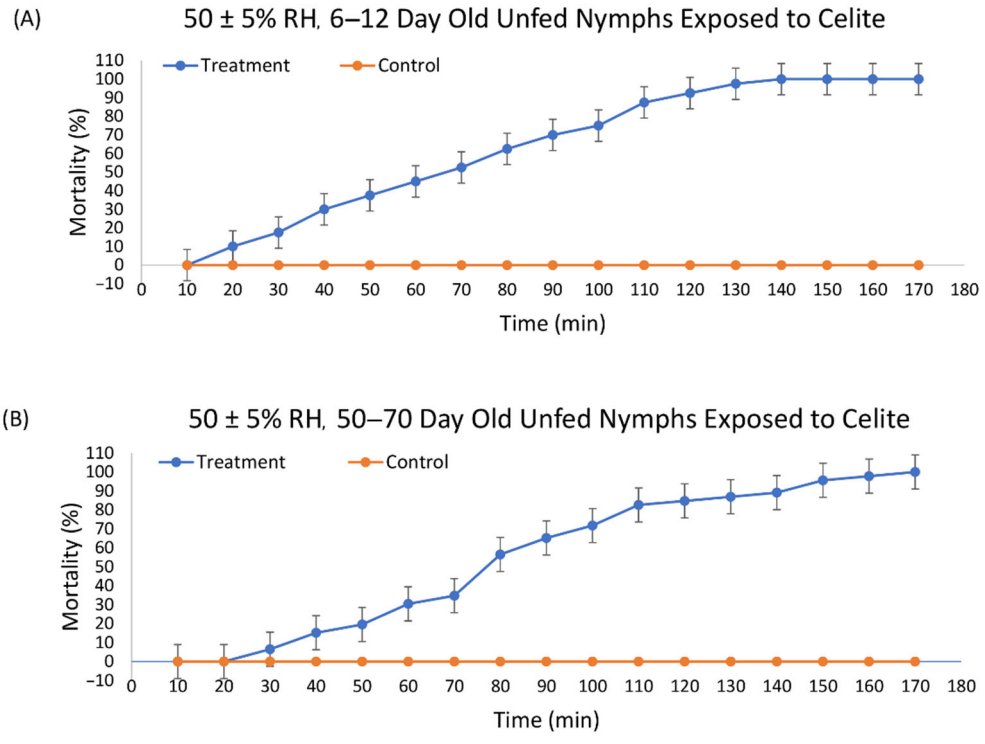


Figure 2. *Cont.*

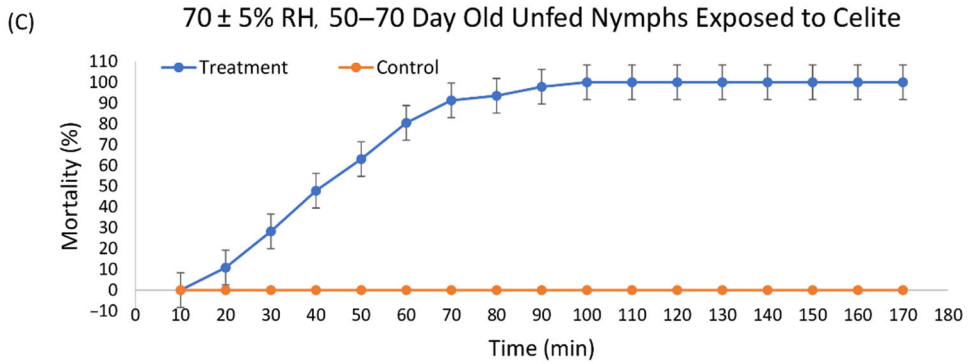


Figure 2. Mortality over time of unfed nymphal *Ixodes scapularis* exposed by dipping into Celite 610 with (**A**) ticks 6–10 days past last molt at 50 ± 5% relative humidity (RH), (**B**) ticks at 50–70 days past last molt at 50 ± 5% RH, and (**C**) ticks 50–70 days past last molt at 70 ± 5% RH.

Probit models were used to determine the time to 50% mortality (LT_{50}) and time to 90% mortality (LT_{90}) (Table 1). The LT_{50} for 6–10 d old unfed nymphs at 50% RH was 66.84 min (95% CI 62.12 to 71.43 min). The LT_{90} was 113.42 min (95% CI 106.49 to 122.12 min). Ticks 50–70 d old at the same RH had an LT_{50} of 81.68 min (95% CI 77.44 to 85.86 min) and an LT_{90} of 128.31 min (95% CI 122.26 to 135.53 min). Higher age increased the time to death at the LT_{50} and LT_{90} by 1.2- and 1.1-fold, respectively.

Table 1. Probit model, LT_{50} and LT_{90} of *Ixodes scapularis* unfed nymphs exposed by dipping into Celite 610 and Imergard.

Mineral	RH *	Last Molt (Days)	N	Slope (SE)	LT_{50} † (95% CI ‡)	LT_{90} † (95% CI ‡)	χ2
Celite 610	50 ± 5%	6–10	40	0.028	66.84A (62.12, 71.43)	113.42A (106.49 122.12)	7.20
Celite 610	50 ± 5%	50–70	46	0.027	81.68B (77.44, 85.86)	128.31B (122.26, 135.53)	10.90
Celite 610	70 ± 5%	50–70	46	0.050	43.84C (40.61, 46.93)	69.46C (65.18, 74.89)	5.12
Imergard™ WP	50 ± 5%	50–70	40	0.030	70.40A (65.99, 74.67)	112.56A (106.61 119.78)	17.464

* Values indicate relative humidity, RH (%); † Values are in min; ‡ In each column, lethal rates with overlapping confidence intervals (CI) are not significantly different and are indicated by the same letter.

Ticks incubated at a higher RH died faster (Table 1). The 50–70 d old nymphs at 70% RH had an LT_{50} of 43.84 min (95% CI 40.61 to 46.93 min) and an LT_{90} of 69.46 min (95% CI 65.18 to 74.89 min) (Table 1). Increased RH decreased the time to death at both the LT_{50} and LT_{90} by 1.9- and 1.8-fold, respectively. To confirm mortality, all ticks were found to be dead at 24 h as well. The control ticks remained active and were walking around the assay arena throughout the assays, while the ticks exposed to Celite were obviously less active after dipping. They were quiescent, with some ability to move their legs while laying on their backs, and some were able to crawl.

3.2. Efficacy of Imergard™ WP against I. scapularis

Imergard was also active against 50–70 d old unfed nymphal *I. scapularis* ticks by dipping and incubation at 30 ± 1 °C and 50 ± 5% RH. Mortality first occurred at 20 min with 100% mortality at 160 min (Figure 3). The LT_{50} was 70.40 min (95% CI 65.99 to 74.67) and the LT_{90} was 112.56 min (95% CI 106.61 to 119.78) (Table 1). Imergard was more active than Celite on unfed, 50–70 d old nymphs at 50% RH by 1.2-fold at the LT_{50} and 1.1-fold at the LT_{90}.

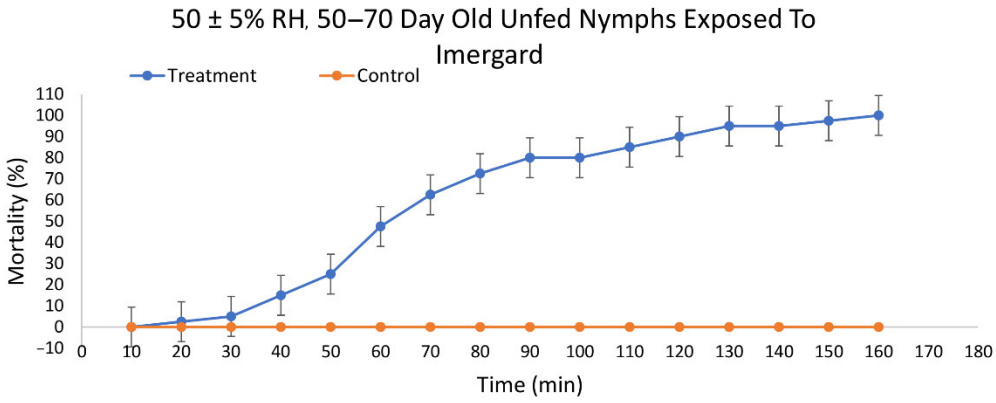


Figure 3. Mortality over time of unfed nymphal *Ixodes scapularis* ticks 50–70 days past last molt exposed to Imergard WP by dipping and incubated at 30 ± 1 °C and 50 ± 5% relative humidity.

3.3. Scanning Electron Microscopy

Scanning electron microscopy was performed on ticks after exposure to Celite and their death (Figure 4). The ticks were fully covered in Celite with variations in the amount between ticks on their dorsum (Figure 4A,B) and from one place to the other on the dorsum (Figure 4B). The capitulum had a similar coating level to the other areas of the dorsum (Figure 4A,C). As shown on the dorsum in Figure 4C, high coating levels were not found on the capitulum. The venter consistently had high levels of coverage, as shown in Figure 4D, and the legs appeared not to display these high levels (Figure 4A,B,D). Figure 5A shows the spiracular plate in the control, which leads to the tracheal (respiratory) system. Figure 5B,C shows that the opening is partially or completely covered, respectively, by Celite. Additionally, no cuticle damage was observed on the dorsum of the ticks (Figure 4A).

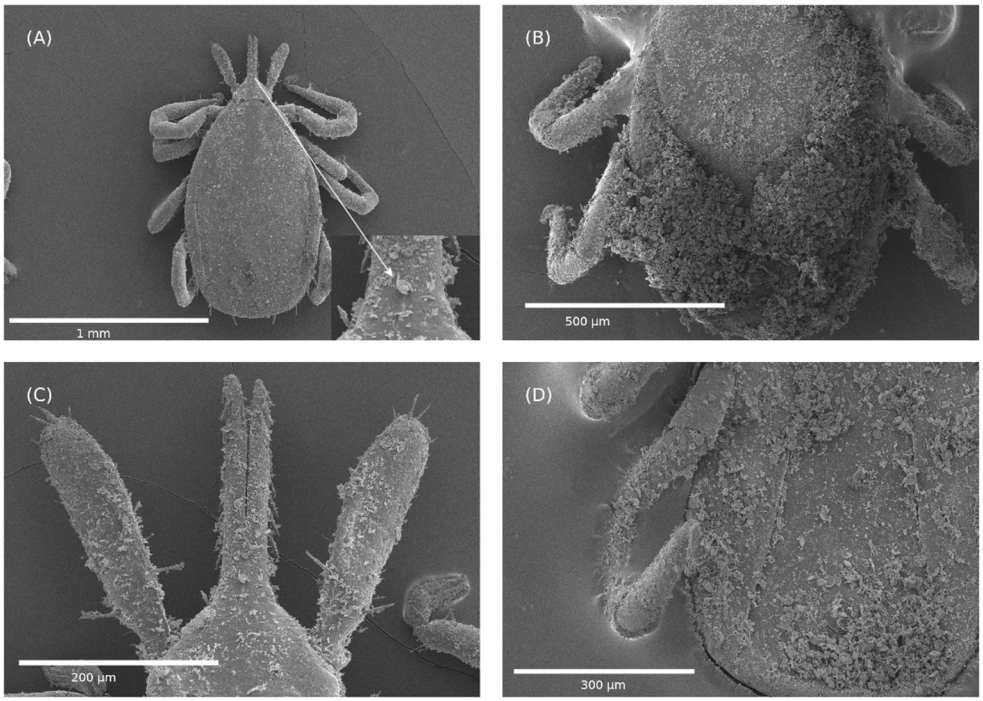


Figure 4. Scanning electron micrographs of *Ixodes scapularis* unfed nymphs exposed to Celite 610 by dipping. (**A**) Dorsal side of the tick, (**B**) close up of the tick on the dorsal side, (**C**) capitulum of the tick, and (**D**) ventral side of the tick, also showing legs. The arrow in (**A**) shows a close-up of a particle of Celite and reveals that there is no cuticle damage around the particle after being exposed to Celite.

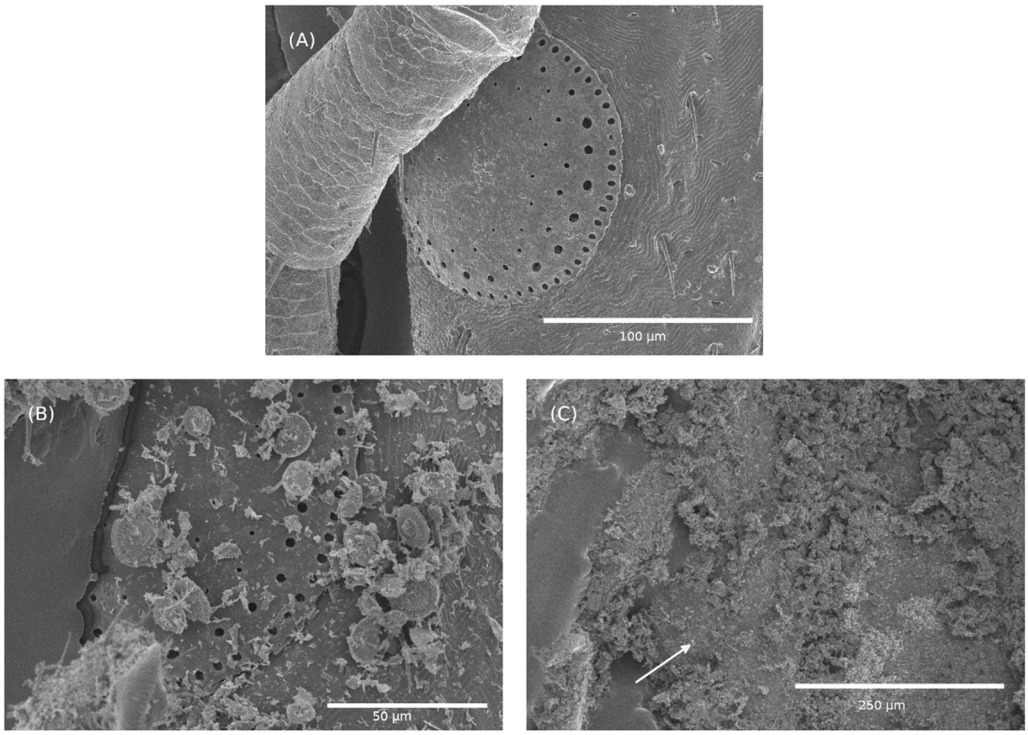


Figure 5. Tick spiracular plate (**A**) untreated and (**B**,**C**) treated with Celite 610. The arrow in (**C**) points to the location of the spiracular plate almost completely obstructed by Celite.

4. Discussion

Celite 610 and Imergard WP demonstrated acaricidal activity against *I. scapularis* unfed nymphs in our dip submersion assay. Ticks 6–10 d past molt exposed to Celite had a shorter LT_{50} of 66.84 min compared to 50–70 d old ticks at 81.68 min (Table 1). This also occurred at the LT90. The age difference in time to death might reflect a lower water permeability across the cuticle and/or the desiccation resistance of the tick in general in the older nymphs, as older nymphs may have a more developed cuticle that provides better protection against desiccation [17,32]. On the other hand, these results were counter-intuitive since the older ticks had a longer time since their last blood meal and might be expected to be more dehydrated than the younger ticks. It is also possible between 6–10 d and 50–70 d that the latter had more time for water to move from the digestive system into the hemolymph and/or the processing of the blood was completed and the ticks were metabolically less active with reduced levels of respiration and associated water loss.

The ticks that were exposed to Celite in high humidity conditions (70% RH) had an LT_{50} of 43.83 min, which is about half the time as the ticks of the same age at 50% RH where the LT_{50} was 81.68 min (Table 1). This result was the opposite of what would be expected for a number of reasons. Normally, high humidity conditions increase tick survivability [33,34]. Ticks are at a constantly high risk of desiccation, especially when host-seeking [35], and the tick's microclimate and especially temperature and humidity affect their locomotory activity, habitat position, and survival [33,34,36–38]. Berger [39] found that low relative humidity reduced *I. scapularis* activity. Ticks have also been shown to change their behavior to avoid desiccation [40,41]. In our assays after treatment, the ticks curled their legs inward and became quiescent, moving less. This could have been a behavioral attempt to prevent further dehydration. Previous studies with insects found that the efficacy of diatomaceous earth (DE) was reduced under high humidity conditions [42–44]. Even slight changes in relative humidity significantly affected the reduced efficacy of DE [42]. Finding the opposite effect of high humidity on ticks is not clear and, along with the rapid reduced activity and mortality after treatment with Celite and Imergard, suggests that another or

additional mechanism of action other than dehydration is occurring. More research is needed to clearly understand the mode of action of these mechanical acaricides on ticks.

The mode of action of mechanical insecticides and acaricides is thought to occur by physical damage to the cuticle, leading to desiccation [21,22]. This is thought to also be the mode of action for Celite and Imergard. However, in our study, we did not observe any damage to the cuticle that could have led to desiccation in our SEM images (Figures 4 and 5). Furthermore, the ticks were not physically active after treatment, suggesting that abrasion was not a factor in cuticle damage. The thick cuticle of a tick was reported to be highly water impermeable [45,46]. Additionally, an approximately two-fold reduction in the time to mortality at 70 versus 50% RH suggests that dehydration is not a mechanism of action. If the disruption of water balance was a factor, we would expect to see ticks die at a slower rate in high humidity. This led us to consider other possible modes of action. Figure 5B shows that the opening into the tracheal system was occluded by Celite, whereas in the case of Figure 5C, there appears to be an almost total blockage. The spiracles of a tick are a critical part of its respiratory system, responsible for the regulation of gas exchange but also limiting water loss from the tracheal system [47–49]. Other studies have found that when the spiracular plate is wet with alcohol, it leads to decreased survival in *Ixodes* ticks [46]. With this in mind, one contributing factor to mortality might be the impact of the mineral on tick respiration.

Ticks exposed to Imergard produced an LT_{50} of 70.40 min at 50% RH (Table 1). This LT_{50} was about 11 min shorter than that for Celite under the same conditions (81.68 min). This technically suggests that Imergard is more effective at killing *I. scapularis*, although practically does not appear to be of major consequence. What is more interesting is that two different mineral types, volcanic glass versus amorphous silica, respectively, are almost equally efficacious. This further supports the idea that the mode of action is physical and not systemic. Understanding the exact mode of action will be critical to studies to improve the efficacy of these minerals. One advantage of Imergard is that it does not contain crystalline silica [17], although the levels in Celite are also considered safe.

The mode of action of Imergard in insect studies so far appears to be dehydration, but how this is accomplished is still unclear. In studies with mosquitoes treated with Imergard, no cuticle abrasion was found and the majority of the mineral was found on the legs [18]. Once mosquitoes came into contact with the mineral, they were no longer active [18], similar to what was seen in this study. Chen et al. [17] concluded that filth flies exposed to Imergard died due to a disruption of water balance. In both mosquitoes and filth flies, the time to 50% mortality increased when the humidity was increased, suggesting that the mechanism of action might be different between insects and ticks.

This study is the first to test the effectiveness of the minerals Celite and Imergard on *I. scapularis*. Our results suggest that both of these minerals could be an alternative method of the control of the blacklegged tick, Lyme disease, and other tick-borne diseases. Both of the active ingredients in these products are considered generally safe [16,20,27]. Future research is needed to assess their use under more practical conditions.

5. Conclusions

In summary, the mineral-based products, Celite 610 and Imergard WP, were found to be efficacious against unfed nymphs of *I. scapularis* in laboratory conditions, making them promising potential acaricides. The mode of action of these industrial minerals is not clear. No damage to the cuticle was observed. However, the minerals obstructed the openings to the respiratory system. It appears these industrial minerals could be suitable alternatives to the currently used synthetic acaricides and, because of their putative mechanical mode of action, used as an additional tool for vector control and to manage tick pesticide resistance.

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PLOS ONE

RESEARCH ARTICLE

Internal and external microbiota of home-caught *Anopheles coluzzii* (Diptera: Culicidae) from Côte d'Ivoire, Africa: Mosquitoes are filthy

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Abstract

Over the past 10 years, studies using high-throughput 16S rRNA gene sequencing have shown that mosquitoes harbor diverse bacterial communities in their digestive system. However, no previous research has examined the total bacteria community inside versus outside of mosquitoes and whether bacteria found on the outside could represent a potential health threat through mechanical transfer. We examined the bacterial community of the external surface and internal body of female *Anopheles coluzzii* adults collected from homes in Côte d'Ivoire, Africa, by Illumina sequencing of the V3 to V4 region of 16S rRNA gene. *Anopheles coluzzii* is in the *Anopheles gambiae* sensu lato (s.l.) species complex and important in the transmission of malaria. The total 16S rRNA reads were assigned to 34 phyla, 73 orders, 325 families, and 700 genera. At the genus level, the most abundant genera inside and outside combined were *Bacillus*, *Staphylococcus*, *Enterobacter*, *Corynebacterium*, *Kocuria*, *Providencia*, and *Sphingomonas*. Mosquitoes had a greater diversity of bacterial taxa internally compared to the outside. The internal bacterial communities were similar between homes, while the external body samples were significantly different between homes. The bacteria on the external body were associated with plants, human and animal skin, and human and animal infections. Internally, *Rickettsia bellii* and *Rickettsia typhi* were found, potentially of importance, since this genus is associated with human diseases. Based on these findings, further research is warranted to assess the potential mechanical transmission of bacteria by mosquitoes moving into homes and the importance of the internal mosquito microbiota in human health.

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Introduction

Insects and other arthropods are found in nearly all habitats, are extraordinarily adaptable, and produce large numbers of progeny in a relatively short time [1]. They are able to transmit bacteria, fungi, viruses, and protozoa by mechanical transmission (physical contact) or by biological transmission (from inside their body to another host) [2, 3]. In biological transmission, some of the pathogens relocate within the body of the vector, moving from the digestive system to the salivary glands; for example, *Rickettsia rickettsii* that causes Rocky Mountain spotted fever, is dormant in the tick's body and activated after the initiation of tick feeding [4]. In mechanical transmission, house flies that live in filth and garbage carry and transmit on their legs and mouthparts, the pathogens for cholera, typhoid fever, and dysentery [5–7]. Acute respiratory syndrome coronaviruses (SARS-CoV) were reported to be mechanically transmitted by insects [8, 9].

Mosquitoes play a significant role in the transmission of diseases like Malaria, Zika fever, Dengue, Chikungunya, and Yellow fever. Of the medically important mosquito species, *Anopheles gambiae* sensu lato (s.l.) [10], largely anthropophilic, is an important malaria vector in Africa; the disease is caused by a parasitic protozoan of the genus *Plasmodium*. Globally in 2020, an estimated 241 million malaria cases were reported [11]. The WHO African Region accounted for 95% of these cases and 96% of the deaths with 85% being children under 5 years old [11].

Vector control is a highly efficacious prevention method for malaria. The main vector control measures are indoor residual sprays (IRSs) and insecticide-treated nets (ITNs). IRSs and ITNs can decrease the vectorial capacity by reducing the adult's longevity, density, and biting rate per day. However, the development of insecticide resistance has decreased their efficacy [12]. Recent survey data showed both *Anopheles coluzzii* (one species of the *An. gambiae* complex) and *An. gambiae* sensu stricto (s.s.) had high resistance levels to pyrethroid, organochlorine, and carbamate insecticides [13]. New strategies for managing resistance and malaria vector control are needed.

In the last 10 years, microbiota studies have been conducted on *Anopheles* mosquitoes from African countries, including Kenya [14–16], Ghana [17], Mali [18], Ethiopia [19], Burkina Faso [16, 20, 21], Cameron [22, 23] and the Republic of Guinea [16]. The bacterial microbiota of *Anopheles* mosquitoes has also been characterized in other malaria-endemic Asian countries, i.e., Vietnam [24], Thailand [25], and India [26]. Several recent microbiota studies on the mosquito midgut have revealed the presence of a diverse microbiome, which can significantly affect the development, digestion, immunity, metabolism, and other physiological functions of the mosquito [27, 28]. Additionally, the microbiome affects the ability of insects to transmit pathogens. For example, the mosquito's midgut microbiota was involved in the suppression of *Plasmodium falciparum* by stimulating the basal immune response of *An. gambiae* s. s. mosquitoes [29, 30]. Although microbiomes have been studied in many different mosquito species, our understanding of bacterial composition and structure in *An. coluzzii* is limited. Further, mosquitoes are exposed to a range of microbes according to their ecological niches like larval habitats [31], outdoor resting sites [32], and plant nectar sources [33]. This could play an essential role in malaria transmission in different environments. Characterizing the bacterial community of *An. gambiae* s. l. in homes in Côte d'Ivoire will improve our understanding of their microbiome in the human domestic environment. Additionally, like with filth flies, *Anopheles* mosquitoes moving from outside into homes, and interacting with surfaces in the home and people directly has the potential of transmitting bacteria mechanically that could be a health concern different from the well recognized biological transmission by mosquitoes. To our knowledge, this is the first study conducted to investigate the external body bacteria of *An. gambiae* s.l. and whether *Anopheles* mosquitoes are potential mechanical vectors.

The objective of the study is to investigate the external and internal body bacterial communities of home-caught adult female *An. coluzzii* using culture-independent methods and providing a basis for future studies characterizing the biological importance of bacteria in this species. Based on previous studies by Deguenon et al. [6], we hypothesize that the bacterial communities from the external and internal body of the mosquito possibly vary, and the bacterial communities from the external surface of the mosquito body between homes would be diverse. Further, we hypothesized that the mosquitoes collected from homes in Africa might harbor pathogenic bacteria not previously appreciated.

Material and methods

Ethics statement

All persons in visited households were informed about the purpose and nature of the study, what participation in the study requires, possible risks and benefits, and verbal consent was obtained and witnessed by signature on a master household list. It was also stressed that any person may refuse dwelling mosquito search/collection at any time without negative consequences. None of the information registered is sensitive. The master household list included a unique identification # per house. Household names and GPS locations were not recorded. All results of the study were reported blindly.

Mosquito sampling and sample preparation

In June 2019, adult mosquitoes were collected in the municipality of Tiassalé, Africa, in the south of Côte d'Ivoire about 110 km North of Abidjan, one of the country's largest cities [34]. Rice production occurs in the Tiassalé's lowlands, allowing mosquitos to increase all year, and malaria is the primary cause of sickness [34]. Sampling was conducted house-by-house in a five-block area of Tiassalé (5˚53'54" N, 4˚49'42" W; S1 Fig). A total of 80 houses were visited, and all the adult mosquitoes in the living rooms were collected. A total of 79 female mosquitoes were collected from 9 houses (see S1 File; most of the homes did not have mosquitoes). Female mosquitoes were collected using aspirators. Mosquitoes collected from each home were placed in a new 125 mL plastic cup. Less than 2 hours after collection, the samples were transported from the field back to the lab. They were chilled in freezer for about 45 seconds, gently picked up with forceps on one leg, and placed on a microscope slide to identify species based on their morphology [10]. After identification, mosquitos were placed individually into 1.5 ml microcentrifuge tubes containing 200 μL of RNAlater and shipped to the North Carolina State University for DNA analysis. Each mosquito was inspected for intactness (all body parts attached), and then intact mosquitoes were rinsed four times with 200 μL of sterile 0.01 M phosphate-buffered saline (PBS, pH 7.4 at 25˚C). All wash solutions and RNAlater were pooled for each mosquito and stored at -80˚C for later surface microbiome analysis (external body samples). The mosquitoes were then stored in 100% ethanol at 4˚C until further use.

DNA extraction and mosquito molecular identification

Before DNA extraction, each mosquito was sterilized with 1% bleach for 30 s, followed by five separate washes with 200 μL PBS (pH 7.4 at 25˚C). The last wash of each mosquito was kept for further verification of the success of the surface sterilization. After surface sterilization, mosquitoes were transferred individually to sterile 2 mL screw-capped microcentrifuge tubes containing glass beads with 200 μL of TNE buffer (100 mM Tris, 0.2 M NaCl, 10 mM EDTA, pH 7.4) added, and samples were homogenized using a FastPrep FP120 cell homogenizer (Thermo Electron Corporation, Waltham, MA, USA). The external body samples were

lyophilized to dryness and resuspended in 200 μL of TNE buffer. The DNA of internal body and external body samples were extracted separately from each mosquito using the QIAGEN DNeasy Blood & Tissue Kit based on the manufacturers' instructions (QIAGEN, Valencia, CA, USA). DNA quality and quantity were assessed using a Nanodrop (Thermo Fisher Scientific, Waltham, Massachusetts), DNA concentration normalized to 50 ng/μL, and then samples stored at -40˚C until PCR amplification. *An. gambiae* complex mosquitoes species were molecularly identified (M or S form) using amplicons size of Short Interpersed Element (SINE) regions by PCR, using previously published primers [35, 36]. SINE-PCR was amplified using 2 μL of DNA template extracted from individual mosquito, 12.5 μL of AmpliTaq Gold™ 360 Master Mix, 1μL (10 pmol) of primer F6.1A, 1μL (10 pmol) of primer R6.1B, and molecular grade nuclease-free water added to achieve a final volume of 25 μL. The temperature of the PCR reaction was set as follows: 95˚C for 10 min, 35 cycles of 95˚C for 30 s, 54˚C for 30 s and 72˚C for 30 s, then 72˚C for 10 min. Amplified PCR product was visualized in a 1.5% electrophoresis gel stained by ethidium bromide under UV light. To further verify sequence of the PCR products, 10% of the PCR products (eight samples) were chosen randomly and sequenced by Eton Bioscience (Research Triangle Park, NC, USA).

Illumina library preparation and sequencing

A total of 157 16S V3–V4 amplicon libraries (79 internal body and 78 external body) were prepared according to the Illumina metagenomic sequencing library construction workflow. Briefly, universal 16S primers (341F/806R) were used to amplify the hypervariable V3-V4 region of the bacterial 16S rRNA genes [37]. DNA extraction from the pooled last washes yielded no bands and were not submitted for sequencing. The target amplicon and index PCR product was purified using AMPure XP beads (AXYGEN, Big Flats, NY, USA). Additionally, four mock community gDNA standard (D6305, ZymoBIOMICS™, Irvine, CA) amplicon libraries were included as positive controls to determine PCR amplification bias or sequencing error [38]. DNA library concentration was measured with Quant-iT PicoGreen (Molecular Probes, Inc. Eugene, OR, USA). Final libraries were pooled in equimolar amounts. Illumina sequencing (300-bp paired-ends) was performed at the Microbiome Core Facility, School of Medicine, University of North Carolina, Chapel Hill, NC, USA.

Bioinformatics data processing and statistical analyses

Illumina FASTQ files were demultiplexed and quality-filtered (q20) using Quantitative Insights into Microbial Ecology 2 (QIIME2) [39]. Reads were then denoised, and the paired-end reads merged with the chimera removed using the DADA2 plugin [40]. Primer sequences were trimmed (–p-trim-left-f 17,–p-trim-left-r 21), and the forward and reverse sequences were truncated at 290 and 280 nucleotide, respectively, to remove low-quality sequences (–p-trunc-len-f 290, –p-trunc-len-r 280). DADA2 replaces the traditional OTU-picking process, and it models the sequence error and constructs the exact biological sequences in the samples called Amplicon Sequence Variants (ASVs). Sequences were aligned with the *Align-to-tree-mafft-fasttree* pipeline, and a phylogeny tree was constructed with *q2-phylogeny*. Taxonomy classification was performed on representative sequences that were generated from DADA2 using a Naive Bayes classifier from Greengenes [41] 13_8 with 99% sequence similarity to the OTU data set, then trained following QIIME 2 tutorial docs "Training feature classifiers with q2-feature-classifier" (https://docs.qiime2.org/2022.8/tutorials/feature-classifier/). The sequences of the taxa with relative abundance higher than 1% have also been extracted for BLASTn searches against the National Center for Biotechnology Information (NCBI) database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Additionally, the q2–diversity plugin pipeline was used to conduct the alpha and beta diversity analysis (–p-sampling-depth 8244). Alpha diversity (which estimates diversity within samples) was measured by Shannon's diversity index [42], Observed Features and Faith's Phylogenetic Diversity [43]. To examine the effects of internal body versus external body and sampling locations (different homes), the non-parametric Kruskal-Wallis test ($\alpha = 0.05$) was used on alpha diversity. Beta diversity was measured by the weighted UniFrac [44] and Bray Curtis distance [45], and two of the households with only one mosquito each were excluded. Further, two households with three specimens were excluded from Beta diversity analysis to test whether sample size plays a role in statistical power using Unifrac and Bray Curtis. A PERMANOVA test was run to test for statistical differences between the internal body and external body samples, among sampling homes of internal body samples; and the statistical difference among sampled homes for external body samples. EMPeror [46] was used to visualize the principal coordinate analysis (PCoA) plots from the beta diversity analyses. To see if there was a difference in the abundance of common bacterial taxa between the internal body and external body, t-tests (assuming unequal variances, $\alpha = 0.05$) were performed using the JMP® Pro 16 software (SAS Institute Inc., Cary, NC, USA). The ANCOM [47, 48] analysis was performed using QIIME2 to test the differentially abundant taxa across different homes.

Phylogenetic analyses of *Rickettsia* sequences

Approximate phylogenetic relationships were examined for nine *Rickettsia* sequence variants from this study with other *Rickettsia* sequences obtained from the NCBI database by BLASTn analysis (accessed on January 18, 2021). Multiple alignments were performed using the ClustalW program [49]. Phylogenetic trees were constructed using the maximum likelihood (ML) and neighbor-joining (NJ) analyses with the Kimura two-parameter model [50] in MEGA 11 software [51]. Bootstrapping at 1000 re-sampling iterations were calculated for ML and NJ trees.

Results

Mosquitoes identification

The SINE gene (~ 479 bp band) was successfully amplified for all 79 female *An. gambiae* complex mosquitoes. Based on the visualization of PCR products resolved on agarose gels, all PCR products appeared as a single band, and there was no measurable length variation among samples. BLAST results for the sequences showed 99.50 to 99.75% similarity to *An. gambiae* M from Cameroon SINE S200X6.1 (GenBank Accession number EU881873). Thus, all the mosquitoes collected in this study were *An. coluzzii*, formerly known as *An. gambiae* M form [52].

Evaluation of the mock microbial community

The mock microbial community consisted of 8 bacterial species from 8 distinct genera (S1 Table). All the species present in the mock community were identified by ASV assembly and taxonomic classification. In addition, the ASV assembly and taxonomic classification identified an additional bacterial genus, *Methylobacterium*, which was not present in the mock community. Due to their low relative abundance and no other plausible explanations for their detection, we concluded these were likely either contaminant from sample processing or barcode cross talk from sequencing. Thus, *Methylobacterium* was excluded from further analysis.

Data summary of bacterial sequences from *Anopheles coluzzii*

Illumina sequencing of 16S rRNA produced abundant reads for the bacterial communities across different homes where mosquitoes were collected. A total of 34,221,107 sequence reads

were obtained across the 157 (79 internal and 78 external) samples. After quality filtering with the DADA2 algorithm, 17,267,125 reads were obtained with an average of 109,981.69 reads *per* sample and were assigned to 34 phyla, 73 orders, 325 families, and 700 genera. After rarefaction analysis, four samples (three internal and one external) with lower sequencing depth (less than 8244 sequencing reads) were discarded from the diversity analysis.

Alpha diversity

The rarefaction curves of the observed ASVs saturation in all the samples indicated that our sequencing depth was adequate to retrieve most of the taxa present in the samples (Fig 1, S2 Fig for all homes). The alpha diversities measuring the difference between the internal body and external surface wash samples differed significantly. The number of observed OTUs (Fig 2A) was significantly higher in the internal body than in the external surface wash samples (Kruskal-Wallis, $p < 0.001$). The Shannon index (Fig 2B), which takes into account both species richness and evenness, was also significantly higher in the inside (Kruskal-Wallis, $p < 0.001$). When taking the phylogenetic relationships into account, the Faith's phylogenetic

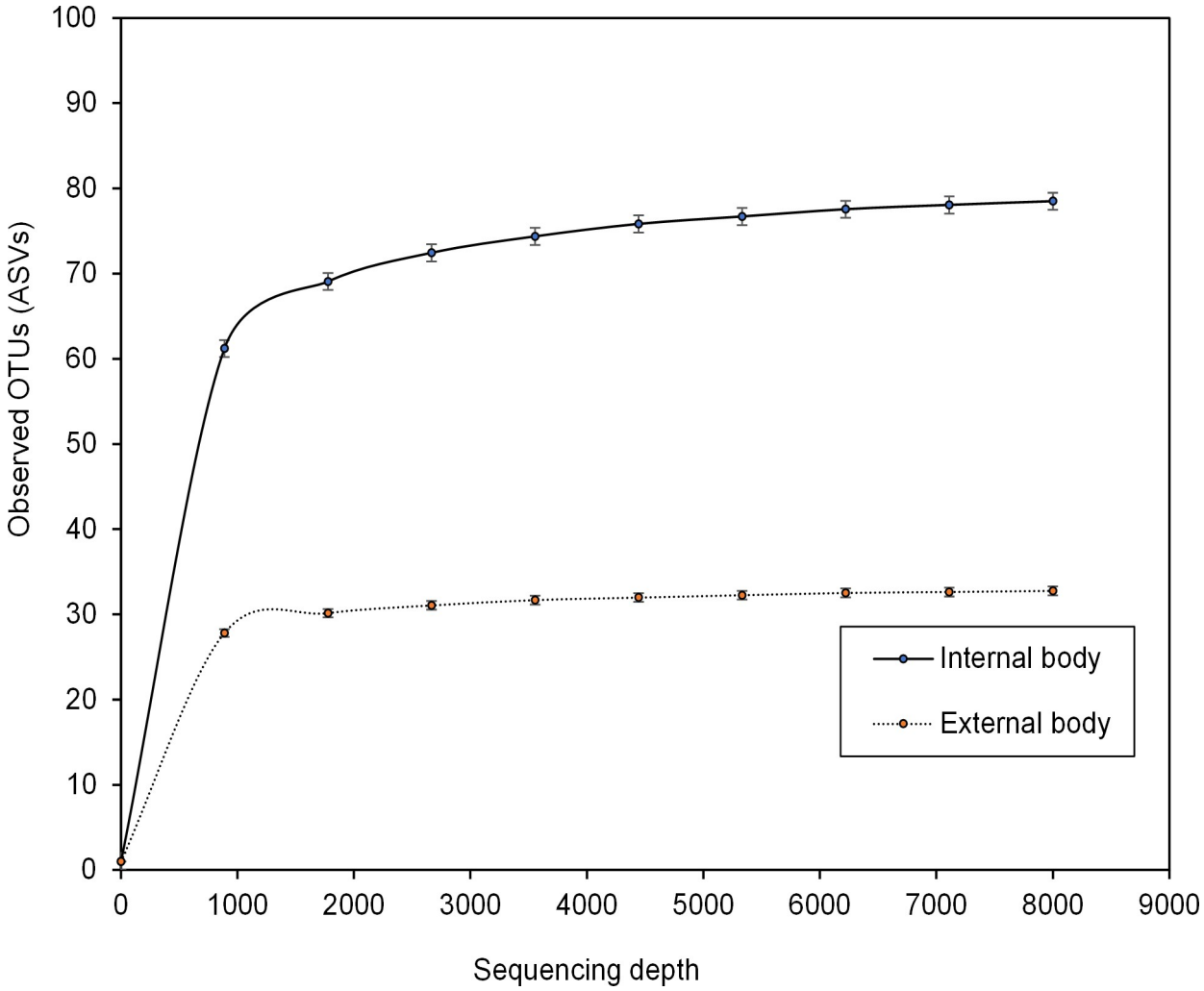


Fig 1. Rarefaction curves of the mean number of observed ASVs in internal versus external body samples.

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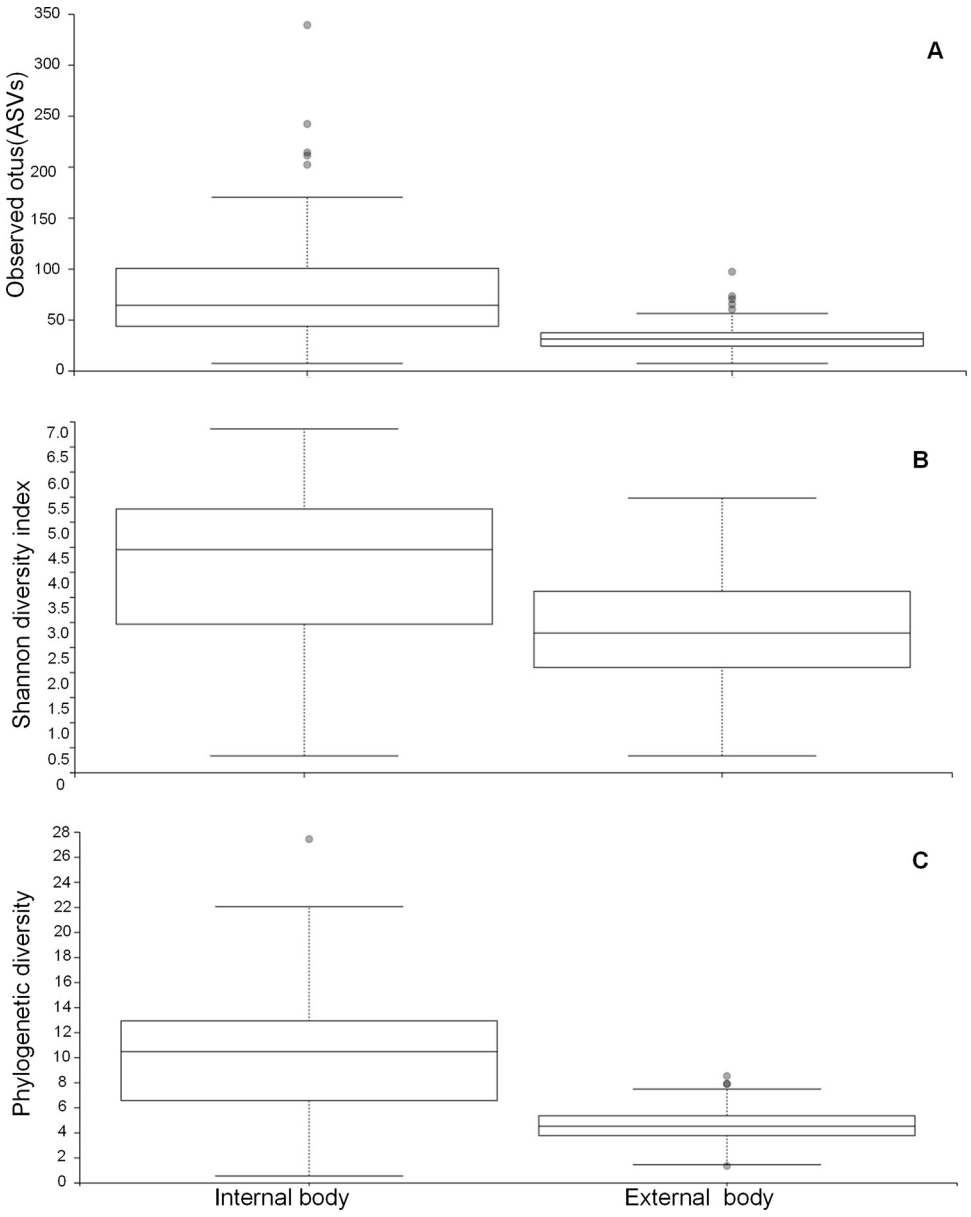


Fig 2. Alpha diversity measures of the internal and external body microbiomes of *Anopheles coluzzii* mosquitoes. (A) Observed OTUs, (B) Shannon diversity and (C) Faith's phylogenetic diversity.

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diversity (Fig 2C) was significantly higher (Kruskal-Wallis, $p < 0.001$) in the internal body samples. Between seven homes (internal body or external body samples), no significant differences (Kruskal-Wallis, $p > 0.05$) were observed (S3 and S4 Figs).

Beta diversity

Weighted Unifrac distance using Principal coordinates analysis (PCoA) (Fig 3) revealed two distinct clusters, the bacterial microbiota on the surface and the internal body of *Anopheles coluzzii* (PERMANOVA, $p = 0.001$). When analyzing the weighted Unifrac distances metric, the bacterial communities in the internal body samples from seven homes were similar (PERMANOVA, $p = 0.11$) (Fig 4). Significant differences were observed when comparing bacterial

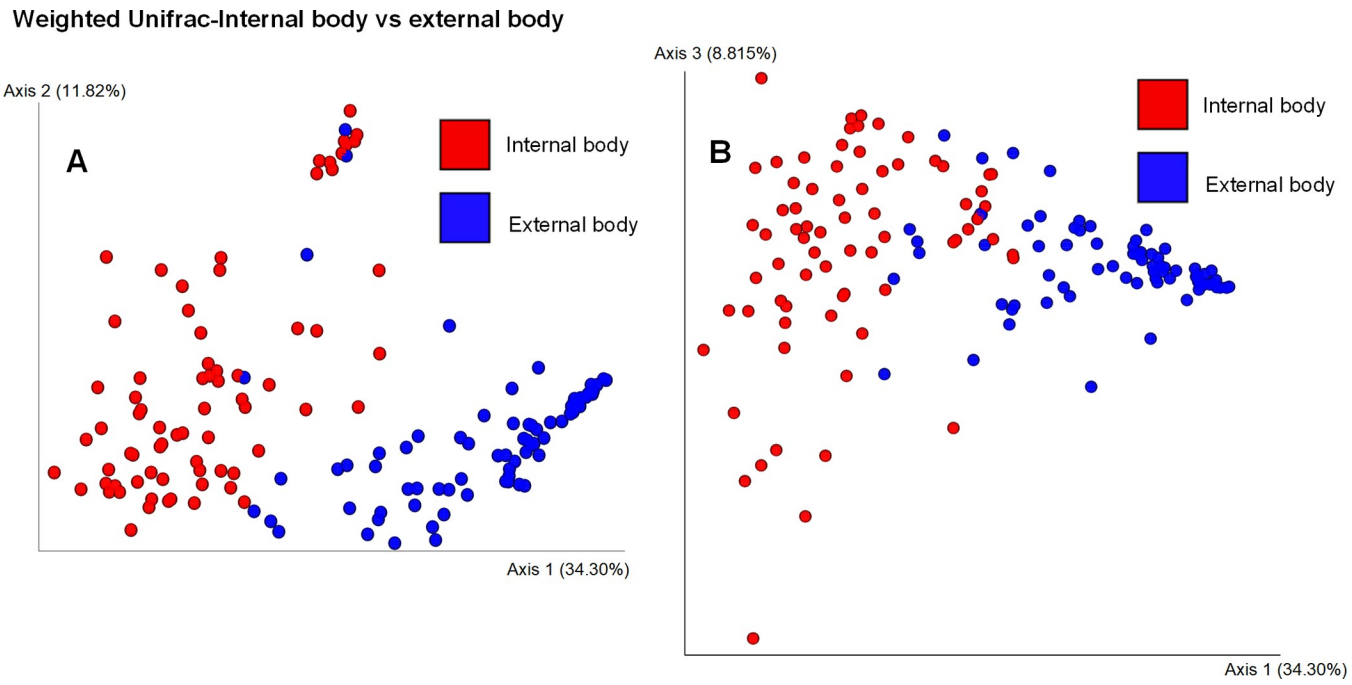


Fig 3. Principal coordinate analysis (PCoA) of bacterial composition between internal and external body samples of *Anopheles coluzzii* mosquitoes. Analysis was based on the weighted Unifrac metric.

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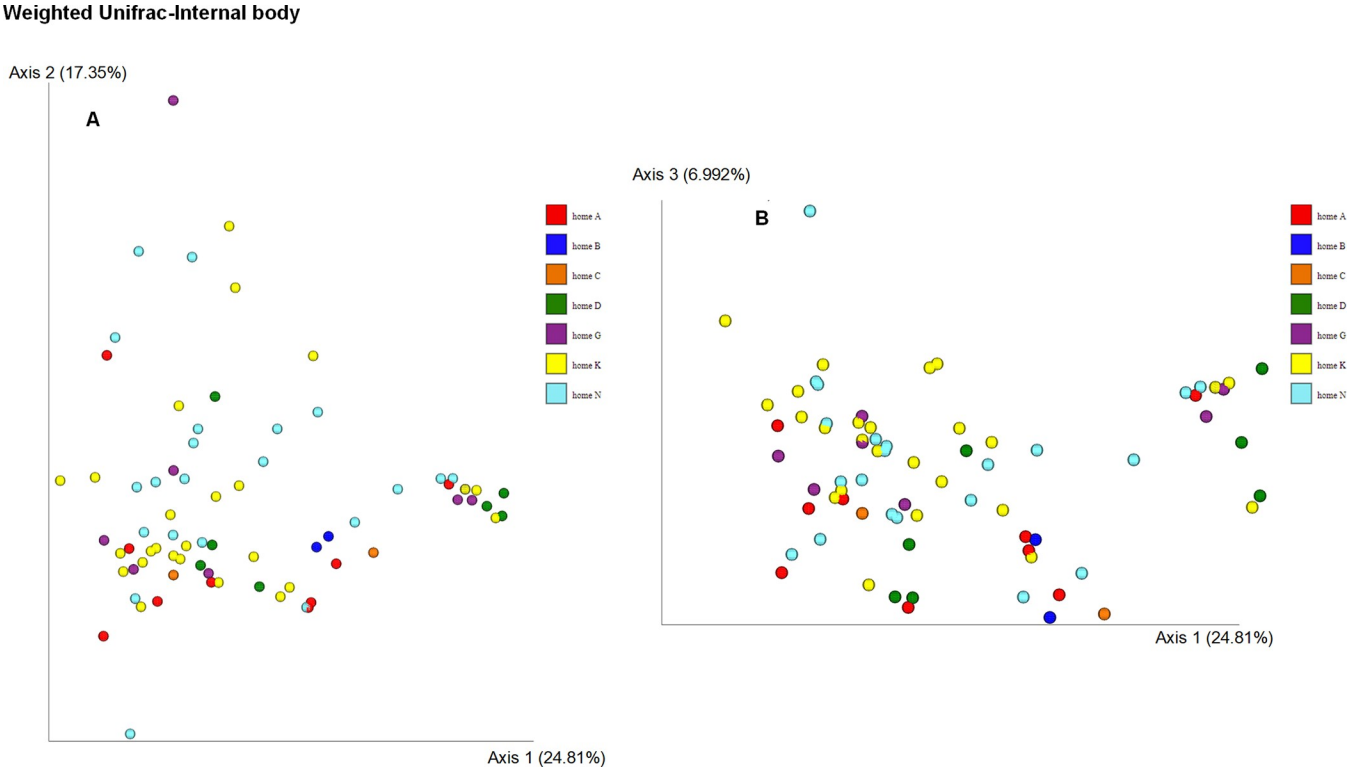


Fig 4. Principal coordinate analysis (PCoA) of the bacterial composition of the internal body of *Anopheles coluzzii* mosquitoes from seven homes. Analysis was based on the weighted Unifrac metric.

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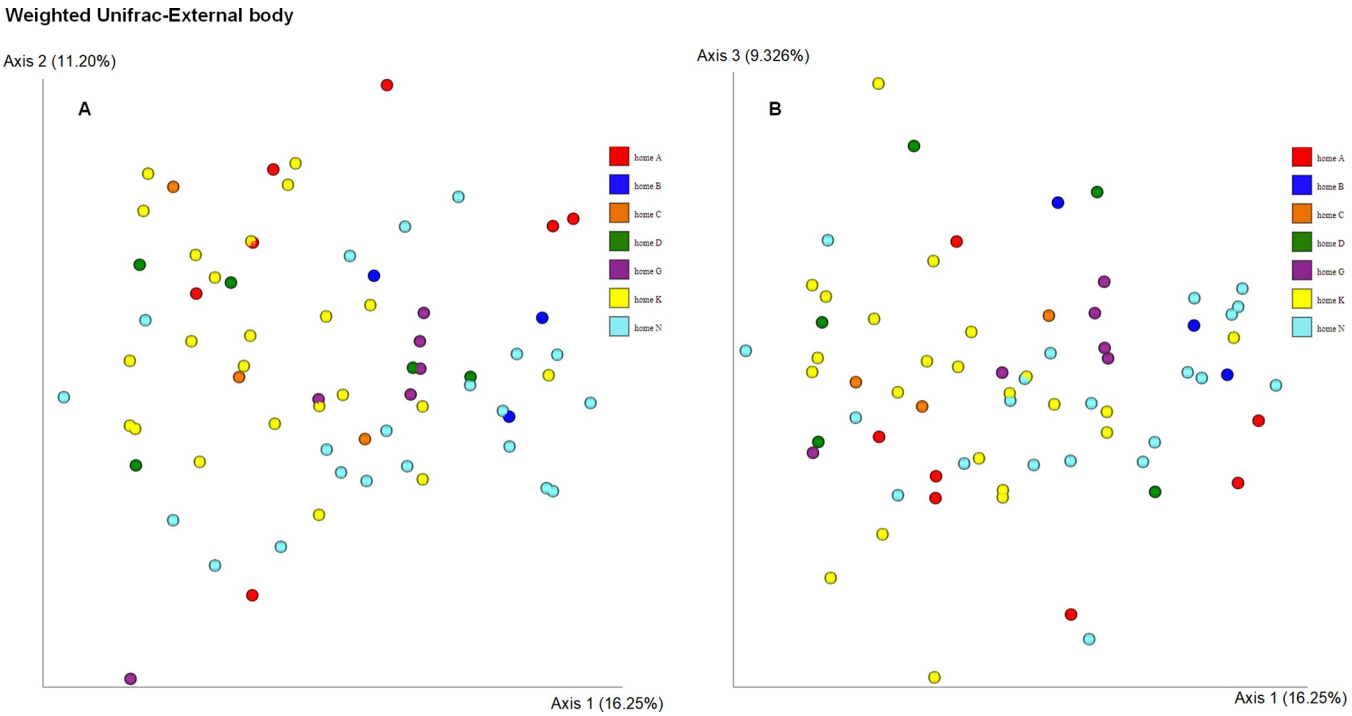


Fig 5. Principal coordinate analysis (PCoA) of the bacterial composition of the external body of *Anopheles coluzzii* mosquitoes from seven homes. Analysis was based on the weighted Unifrac metric.

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communities of external body samples from seven homes using the weighted Unifrac distance metric (PERMANOVA, p = 0.006) (Fig 5). Specifically, significant differences were observed between home A and home G (p = 0.037), home A and home K (p = 0.037), home A and home N (p = 0.037), and home K and home N (p = 0.021).

Non-phylogenetic distance metric Bray Curtis using Principal coordinates analysis (PCoA) (S5 Fig) also revealed two distinct clusters (PERMANOVA, p = 0.001). When analyzing the Bray Curtis distances metric, the bacterial communities in the internal body samples from seven homes were significantly different (PERMANOVA, p = 0.001) (S6 Fig). Significant differences were observed when comparing bacterial communities of external body samples from seven homes using the Bray Curtis distance metric (PERMANOVA, p = 0.001) (S7 Fig). In addition, we analyzed the data without the houses that have three specimens with both Unifrac and Bray Curtis. The results were basically the same; only with the Bray Curtis was there marginal non-statistical significance when the sample size per home was limited to >3.

Bacterial community composition

At the phyla level of taxonomic analysis, four of the most common phyla found across all the samples were Proteobacteria, Firmicutes, Actinobacteria, and Bacteroidetes (S8 Fig). These four phyla accounted for 89.04% of all classified reads in all samples. At the order level, Actinomycetales, Bacillales, Enterobacteriales, Burkholderiales, Lactobacillales, Sphingomonadales, Pseudomonadales and Rhizobiales were predominant in all samples which accounted for 70.7% of all reads. At the family level, Enterobacteriaceae, Bacillaceae, Micrococcaceae, Staphylococcaceae, Sphingomonadaceae, and Corynebacteriaceae were found to be most abundant (S9 Fig). At the level of genus, the most abundant genera identified across all samples were *Bacillus* (5.6% of reads), *Staphylococcus* (4.8%), *Enterobacter* (4.4%), *Corynebacterium* (3.4%), *Kocuria* (2.8%), *Providencia* (2.7%) and *Sphingomonas* (2.5%) (Fig 6).

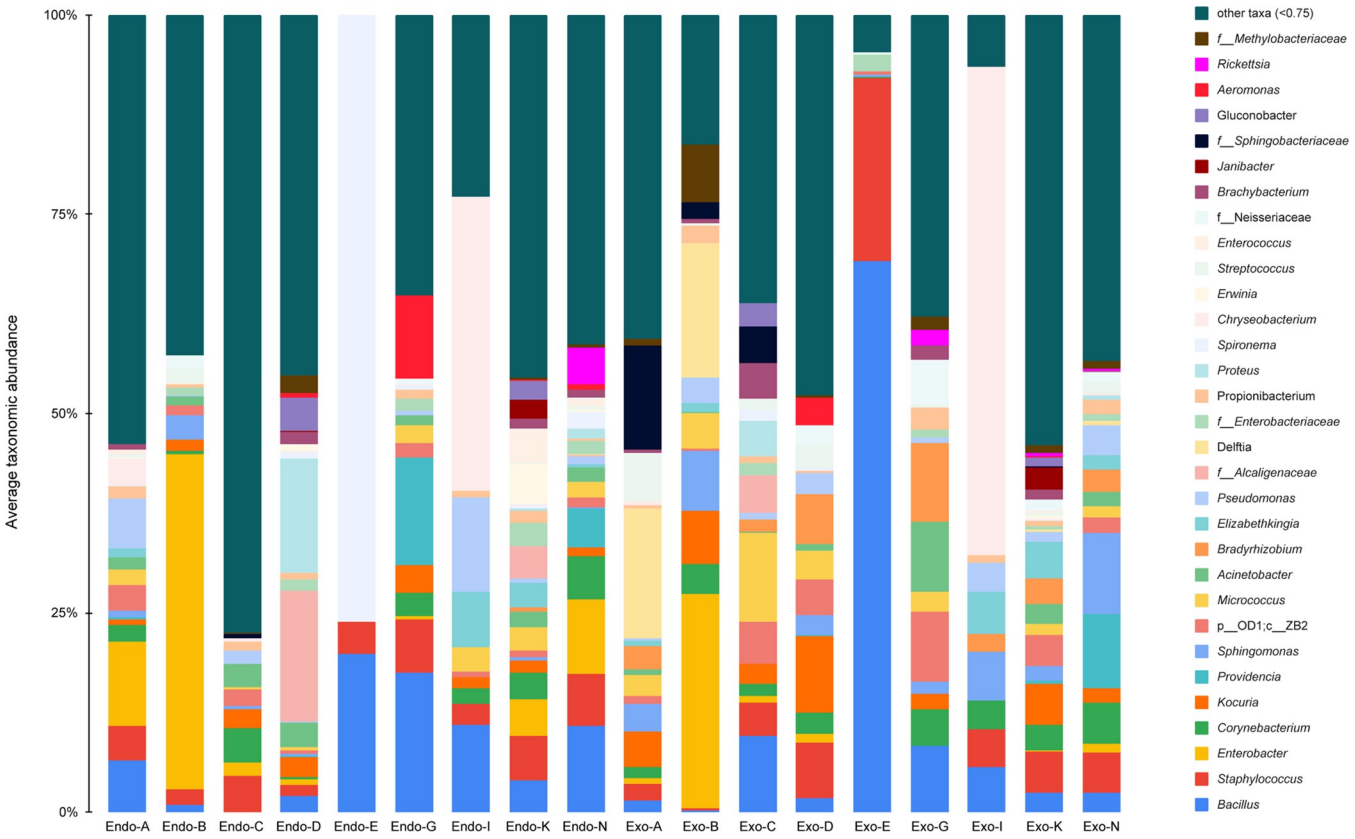


Fig 6. Relative abundances of major bacterial taxa at the genus level. 'Other' group represents all taxa with relative abundance below 0.75%. Each bar plot represents the mean sample composition for each home. Endo represents internal body samples, while Exo represents external body samples. Some taxa were not found at the genus level. "f_" represents family; "p_" represents phylum, and "c_" represents class.

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Internally, the predominant genera were *Bacillus* (7.3% of all internal body reads), *Enterobacter* (7.2%), *Staphylococcus* (5.2%), and *Corynebacter* (3.3%). Externally, the predominant genera were *Sphingomonas* (4.6% of all external body reads), *Staphylococcus* (4.5%), *Kocuria* (4.1%) and *Bacillus* (3.9%). The BLASTn results of the sequences for the taxa with relative abundance greater than 1% are shown in Table 1.

Significant differences (t-tests, $\alpha = 0.05$) in relative abundance for *Enterobacter*, *Kocuria*, *Sphingomonas*, ZB2, *Bradyrhizobium*, and Enterobacteriaceae were found between internal body and external body samples (Table 2). *Enterobacter* and Enterobacteriaceae were significantly abundant in the internal body sample, while *Kocuria*, *Sphingomonas* and *Bradyrhizobium* were significantly abundant in the external body samples (Table 2). *Rickettsia* sequences were detected in internal body samples N23 (95.3%, see S2 File), G6 (0.18%) and A5 (0.004%), and in external body samples G5s (13.5%), K9s (10.8%) and N23s (3.6%).

Differential abundance

The ANCOM test showed that two ASVs from Sphingobacteriaceae and one ASV from *Delftia* significantly differed among different homes in the external body samples (S10 Fig). In 75% of the samples in home C, D, K, and N, one or fewer sequences were observed to have been assigned to *Delftia* (S2 Table). However, in 75% of the samples in home A, 4550.75 or fewer sequences were assigned to *Delftia*, and in 75% of the samples in home B, 6136.5 or fewer sequences were assigned to *Delftia*. The genus *Delftia* was higher in homes A and B than in

Table 1. BLASTn results of the sequences of the taxa with relative abundance higher than 1% in external body or internal body samples.

Taxa classified by Greengeens (External: internal abundance %)	Closest cultured bacteria/ sequence from NCBI (strain)	Classification (Phylum)	Similarity (%)	Closest match NCBI accession #	Habitats
Aeromonas (0.37:1.26)	*Aeromonas hydrophila* (DSM 30187)	Proteobacteria	100	NR_119190	Fresh water and sewage; human pathogen-major bacterial foodborne diseases [99]
Acinetobacter (2.29: 1.83)	*Acinetobacter johnsonii* (ATCC 17909)	Proteobacteria	99.30	NR_117624	Skin [64]
Alloiococcus (1.00: 0.38)	*Dolosigranulum pigrum* (NBRC 15550)	Firmicutes	99.77	NR_113774	Human nasal microbiota [103]
Bacillus (3.92: 7.30)	*Bacillus cereus* (CCM 2010)	Firmicutes	99.77	MT421928	Commonly in the environment; GI syndrome [98]
Corynebacterium (3.54: 3.27)	*Corynebacterium casei* (LMG S-19264)	Actinobacteria	99.76	CP004350	Smear-ripened cheese [84]
Dechloromonas (0: 1.16)	*Azonexus caeni* (Slu-05)	Proteobacteria	99.30	NR_041017	Sludge of a wastewater treatment plant [89]
Delftia (2.64: 0.90)	*Delftia tsuruhatensis* (NBRC 16741)	Proteobacteria	99.77	NR_113870	Sludge of a wastewater treatment plant [90]
Diaphorobacter (1.05: 0)	*Diaphorobacter nitroreducens* (NA10B)	Proteobacteria	99.77	NR_024782	Sludge [88]
Elizabethkingia (2.22: 1.33)	*Elizabethkingia anophelis* (R26)	Bacteroidetes	99.76	CP023401	Midgut microbiota of mosquito [104]
Enterobacter (1.65: 7.16)	*Enterobacter xiangfangensis* (LMG27195)	Proteobacteria	100	CP017183	Chinese traditional sourdough [84]
Enterococcus (0.11: 1.70)	*Enterococcus raffinosus* (1789-79)	Firmicutes	99.77	NR_026499	Human blood culture [105]
Erwinia (0.84:1.80)	*Pantoea agglomerans* (FDAARGOS 1447)	Proteobacteria	100	CP077366	Eucalyptus leaves [80]
Fructobacillus (0.01: 1.30)	*Fructobacillus fructosus* (JCM 1119)	Firmicutes	100	LC062898	Fructose-rich niches [71]
Gluconobacter (0.50:1.15)	*Neokomagataea tanensis* (AH13)	Proteobacteria	99.75	CP032485	Flowers of candle bush [73]
Kocuria (4.06: 1.61)	*Kocuria rhizophila* (TA68)	Actinobacteria	99.51	NR_026452	Skin [67]
Micrococcus (2.30: 2.03)	*Micrococcus luteus* (ATCC 4698)	Actinobacteria	100	CP035298	Human skin flora [63]
Propionibacterium (1.21:1.00)	*Cutibacterium avidum* (ATCC 25577)	Actinobacteria	100	KF906606	Skin [66]
Proteus (0.33:1.75)	*Proteus mirabilis* (ATCC 29906)	Proteobacteria	100	NR_114419	Water, soil, and GI tracts of humans and animals [106]
Providencia (2.82: 2.63)	*Providencia rettgeri* (NCTC 11801)	Proteobacteria	99.77	NR_115880	soil and water;Travelers' diarrhea [100]
Pseudomonas (1.99: 1.46)	*Pseudomonas alcaligenes* (NBRC 14159)	Proteobacteria	99.77	NR_113646	Swimming pool water [107]
Rickettsia (0.36: 1.26)	*Rickettsia bellii* (369L42-1)	Proteobacteria	99.75	NR_036774	Tick [93]
	Rickettsia typhi (Wilmington)	Proteobacteria	99.75	NR_074394	Flea [94]
Sphingomonas (4.61: 0.42)	*Sphingomonas paucimobilis* (FDAARGOS_908)	Proteobacteria	99.75	CP065670	Soil and water [108]
Spironema (0.14:1.84)	*Alkalispirochaeta cellulosivorans* (JC227)	Spirochaetes	83.26	NR_148863	Gut of a wood-eating cockroach [109]
Staphylococcus (4.48: 5.18)	*Staphylococcus haemolyticus* (ATCC 29970)	Firmicutes	100	KT989857	Skin [65]
Streptococcus (2.21: 0.32)	*Streptococcus dysgalactiae* (FDAARGOS_1157)	Firmicutes	100	CP068057	Bovine pathogen [110]
Swaminathania (0.09:1.03)	*Asaia bogorensis* (NBRC 16594)	Proteobacteria	99.75	AP014690	Flowers of the orchid tree (*Bauhinia purpurea*) [74]
Thorsellia (0.15:1.01)	*Thorsellia anophelis* (CCUG 49520)	Proteobacteria	99.77	AY837748	Midgut of the malaria mosquito *Anopheles arabiensis* [111]

https://doi.org/10.1371/journal.pone.0278912.t001

Table 2. Differences in the abundance of common microbiota taxa in internal body and external body samples.

Taxa	Average (± SE) abundance (%)		*df*	*p*
	Internal body (n = 76)	External body (n = 77)		
Bacillus	7.30 (1.76)	3.92 (1.26)	136.26	0.0605
Staphylococcus	5.18 (0.84)	4.48 (0.75)	148.65	0.2678
Enterobacter	7.16 (2.23)	1.65 (1.04)	106.22	**0.0137***
Corynebacterium	3.27 (0.67)	3.54 (0.71)	150.69	0.3888
Kocuria	1.61 (0.49)	4.06 (1.06)	106.58	**0.0193***
Providencia	2.63 (1.68)	2.82 (1.52)	149.26	0.4674
Sphingomonas	0.42 (0.16)	4.61 (1.32)	78.31	**0.0011***
p__OD1;c__ZB2	1.31 (0.23)	3.33 (0.59)	98.59	**0.0009***
Micrococcus	2.03 (0.40)	2.30 (0.65)	127.57	0.3658
Acinetobacter	1.83 (0.40)	2.29 (0.54)	139.40	0.2470
Bradyrhizobium	0.20 (0.10)	3.66 (0.94)	77.66	**0.0002***
Elizabethkingia	1.33 (0.51)	2.22 (0.83)	125.41	0.1808
Pseudomonas	1.46 (0.43)	1.99 (0.53)	144.93	0.2178
f__Alcaligenaceae	2.88 (1.65)	0.18 (0.18)	76.84	0.0547
Delftia	0.90 (0.04)	2.64 (0.96)	76.32	0.0050
f__Enterobacteriaceae	1.71 (0.45)	0.55 (0.23)	112.79	**0.0123***
Propionibacterium	1.00 (0.17)	1.21 (0.38)	104.83	0.3077
Proteus	1.75 (1.35)	0.33 (0.23)	79.46	0.1519

https://doi.org/10.1371/journal.pone.0278912.t002

homes C, D, K, and N among all external body samples. The ANCOM percentile abundance also suggested that the two ASVs from Sphingobacteriaceae were higher in home A than in other homes. In 75% of the internal body samples in homes B, C, D, K, and N, one or fewer sequences were observed to have been assigned to *Dechloromonas* (S2 Table). However, in 75% of the samples in home A, 17395 or fewer sequences were assigned to *Dechloromonas*. This suggests that *Dechloromonas* was higher in home A than in other homes among all internal body samples.

Rickettsia phylogenetic analyses

To further identify *Rickettsia* spp. sequences, nine ASVs that were identified in the genus *Rickettsia*, and 18 closest related species from BLASTn searches were used to construct phylogenetic trees based on the maximum-likelihood (Fig 7) and neighbor-joining (S11 Fig). The phylogenetic analysis suggests that seven of the *Rickettsia* ASVs clustered to *Rickettsia bellii* str. 369L42-1 (NR036774) with an over 99.99% similarity; two of the ASVs clustered to *Rickettsia typhi* str. Wilmington (L36221) with an over 99.99% similarity.

Discussion

In this study, we investigated the bacterial community of the internal body versus external body surfaces of female *An. coluzzii* adults collected from homes in the West African country of Côte d'Ivoire, Africa. Our results revealed that *An. coluzzii* harbor a diverse microbiota, likely influenced by mosquito development, its ecology inside and outside of homes, and how mosquitoes feed. Alpha diversity metrics, the number of observed OTUs, Shannon diversity, and Faith's phylogenetic diversity were all significantly higher in the internal body samples than outside of the insect. The principal coordinates analysis (PCoA) of the beta diversity and weighted unifrac metrics showed two clusters of samples, suggesting that the bacterial microbiota on the surface and internal to the body of *An. coluzzii* were significantly different. Similar

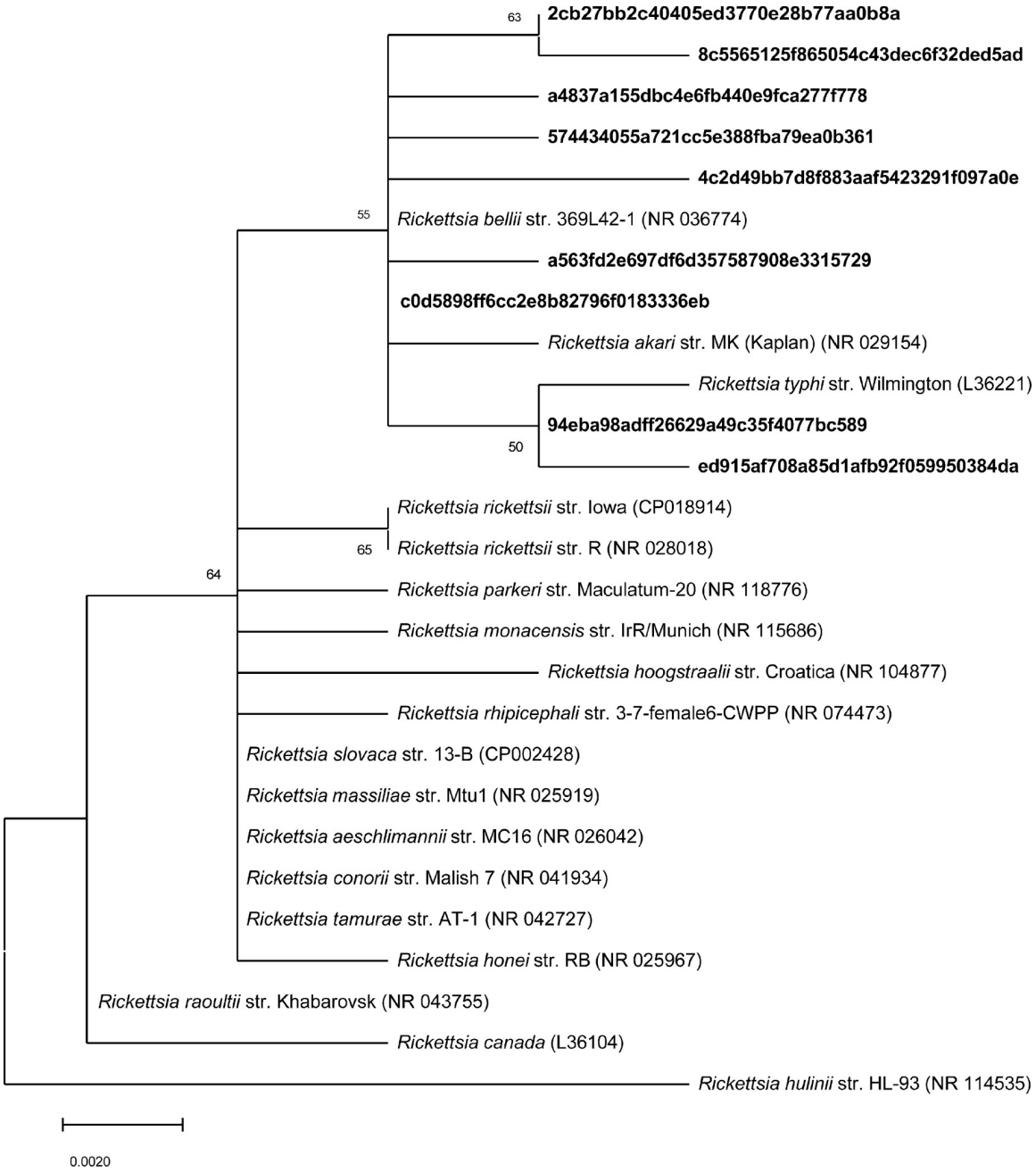


Fig 7. Molecular phylogenetic analysis of *Rickettsia* amplicon sequence variants (ASVs) identified in this study (in bold text) and closest related species. Phylogenetic tree constructed by the maximum likelihood method based on 16S rRNA gene. The percentage of replicate trees with 50% cutoff value in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown below the branches.

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comparisons in other insects are limited. Deguenon et al. [6] compared the internal and external body microbiota of blow flies trapped on an urban farm on our NC State University campus. Park et al. [53] conducted similar work for house flies collected from a farm, home, hospital, and laboratory colony. These two studies found the external body bacterial population was more diverse than the internal population in most cases, which was contrary to our work here with mosquitoes [53]. Exceptions for the house fly research of Park et al. [53] were

observed for insects obtained from a hospital and laboratory colony, where no significant differences were found based on alpha diversity; the mean value of the Shannon diversity and observed ASVs were higher internally than externally for the laboratory house flies.

Our working hypothesis based on earlier studies with blow flies [6] was that bacterial diversity would be greater on the outside compared to the inside. Bacteria found inside have to be adapted to the internal structure and physiology of the insect and resist its natural immunity. Bacteria on the outside are mostly a function of physical interactions with surroundings. Furthermore, blow flies and house flies, might have greater bacterial diversity on their surface than mosquitoes for several reasons. For example, *Anopheles* larvae live mostly in rice paddies, edges of rivers, and streams in the rainy season of Côte d'Ivoire [54], while filth fly larvae live in bacteria-rich environments, e.g., garbage, waste and manure and are adapted to a saprophagous diet [55]. Also, there are significant differences in the external morphology between filth flies and mosquitoes that might favor the mechanical transfer of bacteria to the surface of filth flies. For example, setae size and density and tarsal pads are different between filth flies and mosquitoes. The longer legs and resting behavior of mosquitoes also might reduce the mosquito body from touching surfaces. Blow flies and house flies are stronger fliers than mosquitoes, they interact with different substrates in their habitat with a greater shear force than mosquitoes and move more frequently and at greater distances [56, 57].

Mosquitoes differ from filth flies because they feed on blood (which is essentially sterile). The mosquitoes in our study were collected inside of homes early in the morning. Based on visual inspection at the time of mosquito homogenization, it appears the insects did not contain blood (no appearance of red color). Mosquitoes immediately after blood feeding become quiescent within a short distance from where they obtained their blood meal and during this quiescent period, they digest the blood meal; our collection method would favor the collection of these mosquitoes. Gusmão et al. [58] and Wang et al. [14] reported that bacterial abundance in *An. gambiae* and *Aedes aegypti* increased dramatically after blood feeding. The bacterial load peaked at 48h post blood-feeding, with high levels in the posterior midgut, peritrophic matrix, and around the blood meal, suggesting that bacteria are either benefiting from the nutrients released in these digestive areas and maybe helping to digest blood [58]. Sharma et al. [59] found that the salivary gland of *Anopheles culicifacies* harbor a more diverse microbial community than the digestive system. Our findings of a greater bacteria diversity inside of the mosquito is likely resulting from a combination of factors including differences in habitat, morphology, behavior and feeding differences from filth flies, where bacteria diversity was greater on the outside. More research is needed to obtain a better understanding of this question.

We also observed significant differences in the external body microbiota between homes which was not the case for the internal body samples. These results support the hypothesis that internal bacteria diversity is less affected by environmental exposure compared to what is found on the outside of mosquitoes. Mosquitoes generally are not considered to be filth flies, but at least the potential is there that when they migrate into homes seeking a blood meal, they could also be transferring bacteria from their habitat outside of homes. This will be discussed in more detail later. Mosquitoes also are likely acquiring different bacteria specific to the home. Dunn et al. [60] sampled nine distinct locations in forty homes in Raleigh and Durham, North Carolina, US and found significant bacteria variability between homes. The surface materials, human and animal occupants, the size and cleaning frequency of the home, and indoor humidity and temperature are attributed to this bacterial diversity across homes [60]. This could explain the variability in the bacteria microbiota on the surface of mosquitoes between homes in our study. It is not surprising that the outside surface bacteria microbiota is affected by the mosquito habitat and also suggests that mosquitoes could potentially mechanically transfer human and animal pathogens. The latter has been understudied.

Proteobacteria, Firmicutes, Actinobacteria and Bacteroidetes (from highest to lowest abundance) were the most dominant phyla both internally and externally in our mosquito samples from homes in Côte d'Ivoire. In internal microbiome studies of other dipterans, the order of the latter three phyla varied. However, Proteobacteria was always the most predominant phylum. This was the case for the *An. gambiae* complex mosquito midgut microbiome in Burkina Faso [21], eight *Anopheles* species in Thailand [25], house flies [61], blow flies [6], and fruit flies [62].

BLAST searches were conducted for all bacterial genera that accounted for more than 1% of the internal or external body reads. We identified *Staphylococcus haemolyticus* (external vs internal abundance 4.48%: 5.18%, respectively), *Kocuria rhizophila* (4.06%: 1.61%), *Acinetobacter johnsonii* (2.29%: 1.83%), *Micrococcus luteus* (2.30%: 2.03%), and *Cutibacterium avidum* (1.21%: 1.00%) found on human skin [63–67]. *Staphylococcus haemolyticus* is involved in nosocomial infections, is the second most common coagulase-negative *Staphylococcus* isolated from clinical sites [68], and is also known to be multidrug resistant [69]. Interestingly, a recent study showed that *Staphylococcus* was found to be more abundant among skin microbiota of women who are highly attractive to *An. coluzzii* than poorly attractive group [70]. It is possible that the bacterial genera *Staphylococcus* detected in this study could be acquired by female mosquitoes when feeding.

In this study, several plant-associated bacteria species of *Fructobacillus fructosus* (external vs internal abundance–0.01%:1.30%, respectively), *Neokomagataea tanensis*(0.50%:1.15%), *Asaia bogorensis* (0.09%:1.03%) and *Pantoea agglomerans* (0.84%:1.80%) were identified and were more abundant inside versus outside of our mosquitoes. This is the first identification of a *Fructobacillus* species inside of a mosquito. *Fructobacillus fructosus* is a member of the fructophilic lactic acid bacteria group found in fructose-rich niches [71], for example, in flowers, fruits [72], honeybees and beehives. Plant nectar is an essential source of female mosquito nutrition. Female mosquito immediate mortality due to sugar deprivation does not occur but does reduce fecundity potentially from the reduced production of juvenile hormone [33]. *Neokomagataea tanensis* was first isolated from lantana flowers and candle bush in Thailand in 2011 [73]. The acetic acid bacterium, *Asaia bogorensis*, was first isolated from the flower nectar of the orchid tree, *Bauhinia purpurea* [74]. This bacteria was subsequently found in *Anopheles* mosquitoes and reported to impact larval development [75]. The discovery of the natural Asaia/*Anopheles* mosquitoes/flower nectar cycle was recently considered a possible delivery tool for *Asaia*-based paratransgenetic malaria control [76–78]. *Asaia* was also reported to likely have an indirect role in reducing the vectorial capacity of *Anopheles* mosquitoes by inhibiting the *Plasmodium* sporogenic cycle [76]. In other studies, the genus *Asaia* was reported to be dominated in deltamethrin insecticide susceptible *An. coluzzii* collected from Agboville, Côte d'Ivoire [79]. Our findings of these three bacteria associated with plant nectar suggest the *An. coluzzii* mosquitoes in our samples visited plants for nectar before entering the homes. *Pantoea agglomerans* also are associated with plants [80] and found associated with honey bees [81], aphids, and mosquitoes [82]. Riehle et al. [83] suggested *P. agglomerans* was a good candidate for paratransgenic control of *Plasmodium*.

Two bacteria species, *Corynebacterium casei* and *Enterobacter xiangfangensis*, typically associated with fermented food were identified in our mosquito samples. *C. casei* were found at approximately the same levels external vs internal (3.54%:3.27%, respectively). *En. xiangfangensis* (1.65%:7.16%) were found significantly higher internally. *C. casei* and *En. xiangfangensis* were reported before in smear-ripened cheese [84] and traditional Chinese sourdough [85], respectively. Peach et al. [86] reported that host-seeking mosquitoes were strongly attracted to homemade cheese. *An. gambiae* also is attracted to sugar-fermenting yeast because of the carbon dioxide produced by yeast [87]. *C. casei* and *En. xiangfangensis* identified in our samples,

suggested the *An*. *coluzzii* mosquitoes might have visited fermented foods in the homes where they were found.

Three bacteria species, *Delftia tsuruhatensis* (external vs internal abundance–2.64%: 0.90%, respectively), *Diaphorobacter nitroreducens* (1.05%:0%) and *Azonexus caeni* (0%:1.16%) are associated with aquatic habits where mosquito larvae live. These three bacteria were all isolated from treatment plant wastewater sludge [88–90]. Zogo et al. [54] reported *Anopheles* larvae live mostly in rice paddies in Côte d'Ivoire. Rice paddies are flooded with water producing anaerobic conditions similar to sludge in wastewater. *Delftia* was found both internally and externally and was identified higher in homes A and B than in the other homes among all external body samples (S10 Fig). The relative abundance of *Delftia* in external body samples versus internal samples was 55.7% and 0.4%, respectively; for home B, 35.3% of the reads were identified as *Delftia* in external body samples while no reads were found in the internal samples (see S2 File). This suggests that mosquitoes in homes A and B have been to *Delfti*a rich environments or the larval stages have acquired *Delftia* by feeding and transmitted transstadially to adults. *Diaphorobacter* was only found in external samples. *Azonexus* was found in internal samples and was only detected in samples in home A. This suggests the larval habit of mosquitoes in home A contained this unique bacterium. It is possible that *Azonexus* was transferred transstadially from the mosquito larvae to the adult. The other possibility is the bacteria was acquired from the larval habitat from the consumption of water after emerging from the pupa.

Rickettsia sequences were detected in three internal sample, N23 (relative abundance-95.3%), G6 (0.18%) and A5 (0.004%) and three external samples, G5s (13.5%), K9s (10.8%) and N23s (3.6%). Even though the number of samples detected with *Rickettsia* sequences were the same, the number of reads in the internal samples (147,884 reads) were more than in the external samples (5,244 reads). It is surprising that *Rickettsia* spp. sequences were detected outside of the body since they are intracellular bacteria. The mosquitoes were intact when washing was conducted with PBS buffer to obtain the outside bacteria community. It is possible that pieces of leg or setae or maybe internal content from a severed body part or the rectum separated from the rest of the insect during washing which was not noticed. Further, no obvious defecation was noticed, but it is theoretically possible that gut contents could leak out through the anus even for an insect that is dead. This seems unlikely, especially considering the Illumina sequencing protocol is not nearly as sensitive as PCR, nested PCR in detecting specific bacteria DNA, and requires a robust level of bacterial genomic DNA to detect bacteria. It is also possible that the *Rickettsia* spp. sequences were from ectoparasitic mites that parasitize mosquitoes [91]. It was shown before that 36.45% of *Anopheles* mosquitoes were parasitized with mites [91]. We did not anticipate this possibility and did not search for mites on our mosquito samples.

The closest match of *Rickettsia spp*. sequences using BLAST searches were *Rickettsia bellii* 369L42-1 with a 99.75% similarity (NR_036774) and *Rickettsia typhi* Wilmington with a 99.75% similarity (NR_074394). *Rickettsia bellii* was found in mosquitoes in China, and 2.3% (70/3051) of the sampled mosquitoes, including one *Anopheles* species, were infected with *R. bellii* [92]. *Rickettsia bellii* was widely distributed in multiple tick species in the United States [93]. *Rickettsia bellii* is the ancestral group-*Rickettsia* species, and it could play a crucial role in the ecology and epidemiology of other pathogenic tick-borne spotted fever group *rickettsiae* [93]. *Rickettsia typhi* is the causative agent of murine typhus, also known as endemic typhus [94]. *Rickettsia typhi* can be transmitted to humans by bites and feces of infected fleas. Infected people can have symptoms like a fever, rash and bronchitis and produce severe disease in vulnerable populations like the elderly and immune-compromised people. If left untreated, murine typhus can be fatal, with a 4% death rate [95]. *Rickettsia typhi* was not reported before

in mosquitoes. However, *R. felis*, reported to be the possible causative agent of murine typhus [96], was found in *An. gambiae* from Sub-Saharan Africa [97].

Noteworthy, several food borne pathogenic bacteria were also found both externally and internally. *Bacillus cereus* were more abundant among all bacteria in the internal samples (3.92%:7.30%, respectively). Of the mosquitoes collected, *B. cereus* was found in 67.1% (51/76) of the internal samples and 31.2% (24/77) in the external samples (see S2 File). *Aeromonas hydrophila* (0.37%:1.26%) were more abundant in the internal samples and found in 22.4% (17/76) of the internal samples of the mosquitoes collected and 2.6% (2/77) for the external samples (see S2 File). *Bacillus cereus* is a pathogen associated with food poisoning, diarrhea and other gastrointestinal disorders [98]. *Aeromonas hydrophila* is frequently found in fresh water and sewage and can cause *Aeromonas* enteritis and is a major bacterial foodborne disease [99]. *Providencia rettgeri* (2.82%: 2.63%) was found both internally and externally. *Providencia rettgeri* is associated with traveller's diarrhea [100,101]. Recent studies found *P. rettgeri* in stool samples from diarrhea patients and also in meat samples, suggesting *Providencia* infection in humans could come through meat [102]. These results suggest that they coud be transferred to surfaces in the home by both mechanical interations and defecation and potentially cause food borne diseases to humans. The role of mosquitoes as a filth fly is an understudied area of science and needs more research to assess their risk to humans.

Conclusions

Our study provides the first study of bacterial communities from the internal body and external body of home-caught mosquitoes, i.e., *An. coluzzii*. The mosquitoes had a greater diversity of bacterial taxa internally than externally. The internal bacterial communities were similar between homes, while the external body samples were significantly different between homes. The bacteria on the external body were associated with plants, human and animal skin, and human infections. To our knowledge, Fructobacillus was identified in the internal body of mosquitoes for the first time. Internally, *R. bellii* and *R. typhi* were found, potentially of importance since this genus is associated with human and animal diseases. Based on these findings, further research is warranted to assess the potential mechanical transmission of bacteria by mosquitoes into homes as they seek a blood meal and the importance of their internal microbiota in human health.

Supporting information

S1 Table. Bacterial mock community expected from ZymoBIOMICS™ Microbial Community DNA and actual taxonomic abundances in our samples.
(DOCX)

S2 Table. Significantly differing taxa from ANCOM differential abundance testing resulted and the percentile abundance of taxa by group.
(DOCX)

S1 Fig. Map of areas where mosquitoes sampling was conducted house-by-house in a five-block area of Tiassalé, Côte d'Ivoire, Africa (5˚53'54" N, 4˚49'42" W).
(TIF)

S2 Fig. Rarefaction curves of the mean number of observed ASVs from seven homes.
(TIF)

S3 Fig. Alpha diversity measures of the internal body microbiomes of *Anopheles coluzzii* mosquitoes from seven homes. (A) Observed OTUs, (B) Shannon diversity and (C) Faith's

phylogenetic diversity.
(TIF)

S4 Fig. Alpha diversity measures of the external body microbiomes of *Anopheles coluzzii* mosquitoes from seven homes. (A) Observed OTUs, (B) Shannon diversity and (C) Faith's phylogenetic diversity.
(TIF)

S5 Fig. Principal coordinate analysis (PCoA) of bacterial composition between internal and external body samples of *Anopheles coluzzii* mosquitoes. Analysis was based on the Bray Curtis metric.
(TIF)

S6 Fig. Principal coordinate analysis (PCoA) of the bacterial composition of the internal body of *Anopheles coluzzii* mosquitoes from seven homes. Analysis was based on the Bray Curtis metric.
(TIF)

S7 Fig. Principal coordinate analysis (PCoA) of the bacterial composition of the external body of *Anopheles coluzzii* mosquitoes from seven homes. Analysis was based on the Bray Curtis metric.
(TIF)

S8 Fig. Relative abundances of major bacterial taxa at the phylum level. 'Other' group represents all taxa with relative abundance below 5%.
(TIFF)

S9 Fig. Relative abundances of major bacterial taxa at the family level. 'Other' group represents all taxa with relative abundance below 0.75%.
(TIF)

S10 Fig. ANCOM differential abundance testing result for (A) external body and (B) internal body. Significantly differing taxa from ANCOM differential abundance testing resulted and the percentile abundance of taxa by group are shown in S2 Table. QIIME2 was used for ANCOM analysis.
(TIF)

S11 Fig. Molecular phylogenetic analysis of *Rickettsia* amplicon sequence variants (ASVs) identified in this study (in bold text) and closest related species. Phylogenetic tree constructed by the neighbor-joining method based on 16S rRNA gene. The percentage of replicate trees with 50% cutoff value in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown below the branches.
(TIFF)

S1 File. Mapping file.
(XLSX)

S2 File. Data file.
(XLSX)

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Communication

Rickettsia felis and Other *Rickettsia* Species in Chigger Mites Collected from Wild Rodents in North Carolina, USA

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Abstract: Chiggers are vectors of rickettsial pathogenic bacteria, *Orientia* spp., that cause the human disease, scrub typhus, in the Asian–Pacific area and northern Australia (known as the Tsutsugamushi Triangle). More recently, reports of scrub typhus in Africa, southern Chile, and the Middle East have reshaped our understanding of the epidemiology of this disease, indicating it has a broad geographical distribution. Despite the growing number of studies and discoveries of chigger-borne human disease outside of the Tsutsugamushi Triangle, rickettsial pathogens in chigger mites in the US are still undetermined. The aim of our study was to investigate possible *Rickettsia* DNA in chiggers collected from rodents in North Carolina, USA. Of 46 chiggers tested, 47.8% tested positive for amplicons of the 23S-5S gene, 36.9% tested positive for 17 kDa, and 15.2% tested positive for *gltA*. Nucleotide sequence analyses of the *Rickettsia*-specific 23S-5S intergenic spacer (IGS), 17 kDa, and *gltA* gene fragments indicated that the amplicons from these chiggers were closely related to those in *R. felis*, *R. conorii*, *R. typhi*, and unidentified *Rickettsia* species. In this study, we provide the first evidence of *Rickettsia* infection in chiggers collected from rodents within the continental USA. In North Carolina, a US state with the highest annual cases of spotted fever rickettsioses, these results suggest chigger bites could pose a risk to public health, warranting further study.

Keywords: *Rickettsia*; chigger mites; North Carolina

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1. Introduction

Chigger mites in the genera *Leptotrombidium* and *Herpetacarus* are known biological vectors of the causative agent of scrub typhus, *Orientia tsutsugamushi*, an obligate intracellular bacterium closely related to the genus *Rickettsia* (Rickettsiales) [1,2]. The disease is a significant source of morbidity and mortality, with an estimated one billion people at risk, and approximately one million cases reported each year [3]. Until recently, scrub typhus exclusively occurred within the so-called 'Tsutsugamushi Triangle', ranging from Pakistan in the west, to far-eastern Russia in the east, and to northern Australia in the south. However, scrub typhus was found recently in the Middle East, southern Chile, and Africa [4,5]. Scrub typhus was also thought to be caused by only one species of *Orientia* (*O. tsutsugamushi*); however, a new species, *Candidatus* Orientia chiloensis, was found in South America, where the first case of scrub typhus was confirmed on Chiloé Island in southern Chile [6,7]. In addition to the scrub typhus pathogen, several other rickettsiae known to infect humans, namely *Rickettsia japonica*, *R. akari*, *R. felis*, *R. conorii*, and *R. typhi*, were detected in chiggers removed from wild rodents in Korea [8]. In China, trombiculid

mites collected from wild rodents were found to harbor several other pathogens, including *Orientia tsutsugamushi*, *Bartonella* spp., and *Rickettsia* spp. [9]. Recently, *Candidatus* Rickettsia colombianensi was also detected in chiggers collected from rodents in Brazil [10]. Despite the public health importance and increasing discovery of chigger-borne human disease outside of the Tsutsugamushi Triangle, the role of chiggers in human infection with rickettsial pathogens in the US is still unknown. Here, we report the identification of *Rickettsia felis* and other *Rickettsia* spp. in chiggers removed from rodents trapped in woodland and field habitats of public lands in North Carolina (NC), USA.

2. Materials and Methods

2.1. Site Description

Eight field sites were sampled, three state parks, three game lands, one community recreational green space (i.e., Clawson-Burnley Park, Boone, NC, USA) and one national forest (Chattahoochee National Forest, in the extreme northeastern corner of Rabun County, GA, USA) (Figure 1). All sites are within the greater southern Appalachian Mountains ecoregion, except Lake Norman State Park in North Carolina, USA. The general vegetation in all the sites was mixed woodland or hardwood forest containing tree species such as: oak, hickory, maple, pine, chestnut, birch, and beech. Some open meadows within the game lands and national forest were sampled, and these were characterized by ferns, herbs, grasses, and young regenerative trees. The forest of Clawson-Burnley Park (CBP) surrounds a mowed athletics field. At the Lake Norman State Park (LNSP), the collection site, was a meadow habitat at the forest edge; this was where *Sigmodon hispidus* were collected.

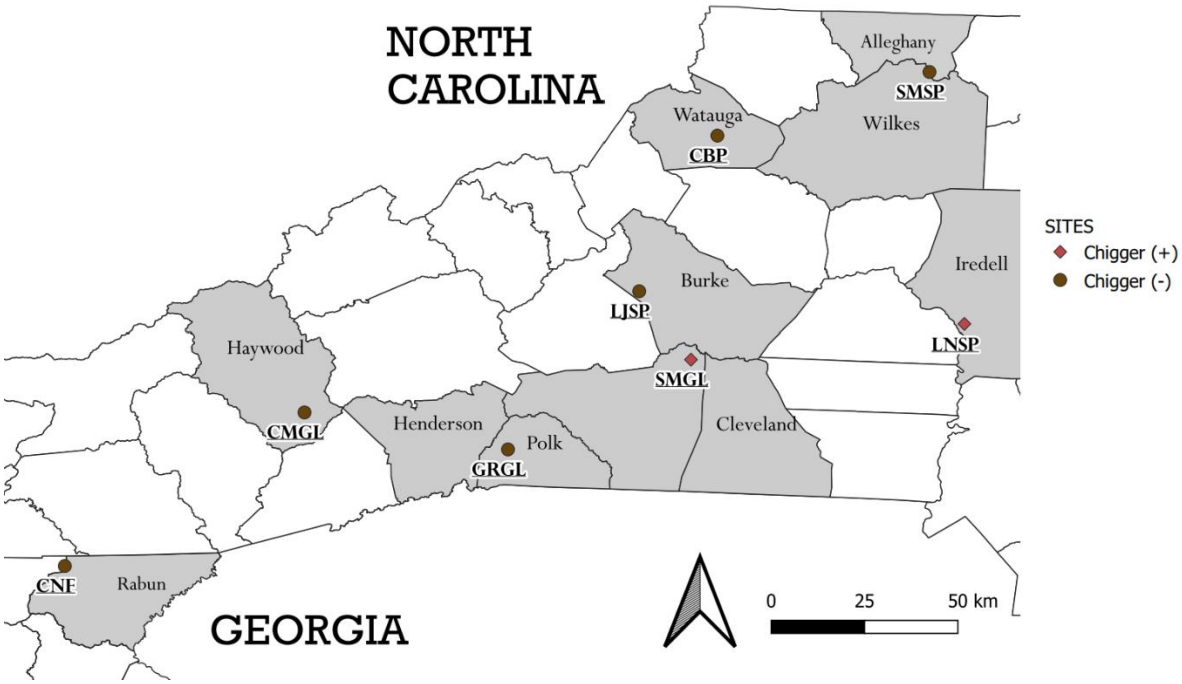


Figure 1. Map of areas where small mammals were sampled for ectoparasites. Shaded counties depict areas where rodents were trapped. Brown circles indicate areas where no chiggers were found on rodents, and red diamonds indicate sites where chiggers were found on some rodents. Some parks span beyond one county (indicated below by asterisks) and trapping extended into the nearby counties. * SMSP—Stone Mountain State Park (Alleghany and Wilkes counties), CBP—Clawson-Burnley Park, LJSP—Lake James State Park, LNSP—Lake Norman State Park, * SMGL—South Mountains Game Lands (Rutherford and Cleveland), CMGL—Cold Mountain Game Lands, * GRGL—Green River Game Lands (Polk and Henderson), CNF—Chattahoochee-Oconee National Forest.

In every site, one or more of the following recreational activities may occur: hiking, kayaking, canoeing, fishing, rock climbing, hunting, birding, biking, camping, and picnicking. Hunting or extraction of other natural resources is only permissible, to variable extents, in the game lands, state forest (not the state parks), and national forest. Picnic-friendly microsites were established in designated places at some sites, either in the woods or open forest spaces, where picnic tables, shelters, and barbecue stands were constructed.

2.2. Rodent Trapping and Chigger Collection

Small mammals were live-trapped (except for one transect at CNF where snap traps were used) from June to September of 2020. Each trapping session consisted of three consecutive nights. Transect lines were established to cover different microhabitats within both forest and meadow. Live-trapping of small mammals primarily involved aluminum Sherman live animal traps (HB Sherman Traps Inc., Tallahassee, FL, USA), Longworth traps (Anglian Lepidopterist Supplies, Norfolk, England); pitfalls were also used at some sites. The use of different trap types increases the overall species richness by improving the chances of trapping species that may be easily caught by one trap type but not the other, and increases the overall capture efficiency [11]. Traps were baited with a mixture of peanut butter (Great Value Creamy Peanut Butter, Walmart Stores Inc., Bentonville, AR, USA) and oats (Great Value Old Fashioned Oats, Walmart Stores Inc., Bentonville, AR, USA). The traps were set at sunset and inspected at sunrise every day of the trapping period, closed after every morning inspection, and reopened again at sunset. Trapped small mammals were either anesthetized using the isoflurane vapor method before ectoparasite screening, or screened under manual restraint without anesthesia. Captured individuals were thoroughly inspected for the presence of chiggers and other ectoparasites by systematically examining the pelage, paying particular attention to the ears, feet, abdomen, and genitals, where blood-feeding ectoparasites are not easily dislodged [12]. Embedded chiggers appeared in clusters as red spots, and were removed using sterile pointed tweezers from their point of contact with the animal's skin, or dislodged from the skin by vigorously brushing the pelage with a louse comb. All chiggers collected were attached to the animal skin. As part of ongoing projects, we also collected fleas, lice, and ticks using the same methods. All collected ectoparasites (including chiggers) were immediately transferred into 1.5-mL Eppendorf tubes or glass vials containing 95% absolute ethanol (Fisher Scientific, Hampton, NH, USA). One vial of ectoparasites was collected per individual small mammal, and these were stored at −20 °C in the laboratory for later identification.

Standard morphometric measurements were taken for each individual small mammal captured (i.e., body length and weight). Animals were also sexed (based on anogenital distance, which is wider in males, or visible evidence of descended testes or enlarged mammae), examined externally for reproductive status, and aged based on pelage and external traits, such as weight. Each animal was identified using the Peterson Field Guide to Mammals of North America, North of Mexico [13]. For uncertain identifications, DNA barcoding was used as a confirmatory test. To provide tissue for DNA barcoding analyses, we either used a 2-mm ear punch kit (Miltex Instruments, York, PA, USA) or subsampled liver tissue from animals collected lethally as part of other ongoing projects. Each tissue type was preserved in 1.5 mL vials containing 95% ethanol. Recaptures within each trapping session (identified by their previous ear punch, weight, transect line, and/or a mark made on the abdomen with a permanent marker) were re-examined for ectoparasites, reweighed, and released at the capture site. Small mammal field handling techniques followed the American Society of Mammalogists guidelines [14] and Institutional Animal Care and Use Committee (IACUC) protocols in place at the University of North Carolina at Greensboro (IACUC 18-002 and 20-008 for Wasserberg and McLean labs, respectively). Permits to conduct trapping at our sites were granted by the North Carolina Wildlife Resources Commission.

2.3. Extraction of DNA from Chigger Samples

To prevent cross-sample contamination, we performed template DNA extraction and PCR setup in separate rooms with dedicated pipette sets, used and certified DNase- and RNase-free filter barrier tips to avoid aerosol contamination. Total DNA was extracted separately from 46 individual chiggers collected from a portion of the *P. leucopus* and *S. hispidus* captured at the Lake Norman State Park and South Mountains Game Lands sites. To remove surface contaminants, including microbes on the surface of a chigger, prior to DNA extraction, each individual chigger was surface sterilized using methods described by Ponnusamy et al. [15]. The DNA of the chigger samples was extracted using the QIAGEN DNeasy Blood & Tissue Kit, based on the manufacturers' instructions (QIAGEN, Valencia, CA, USA). Briefly, after surface sterilization, each chigger was transferred into a separate sterile 2-mL screw-capped microcentrifuge tube containing ~10 sterilized 3-mm glass beads (Cat. 11-312A, Fisher Scientific), with ATL lysis buffer and Proteinase K solution. Next, samples were homogenized using a FastPrep FP120 cell homogenizer (Thermo Electron Corporation, Waltham, MA, USA), and then incubated at 65 °C for 1 h. AL buffer was added to each tube, and the samples were mixed by pulse vortexing, and then incubated at 56 °C for 1 h. DNA was purified according to the manufacturers' instructions. DNA was eluted using 30 µL of nuclease-free water and stored at −20 °C until further processing.

2.4. Identification of Chigger Samples

Molecular identification of chigger samples was conducted by PCR amplification of the mitochondrial cytochrome c oxidase subunit I gene (COI), using previously described primers and amplification protocols [16]. Twenty percent of amplicons were Sanger sequenced at Eton Bioscience, Inc. (Research Triangle Park, NC, USA). Morphologically based identifications were conducted using expert knowledge and published taxonomic keys [17–19]. A portion of the chiggers collected from South Mountains Game Lands were submitted as voucher specimens to the UNCG Parasite Collection. Similarly, Lake Norman State Park chigger subsamples were submitted to the Georgia Museum of Natural History.

2.5. Amplification of Rickettsia spp. from Chigger DNA

We screened the subset of chigger samples by amplifying the 23S-5S intergenic spacer (IGS) [20,21], and the 17 kDa [22] and *gltA* genes [23,24], using the *Rickettsia*-specific primers provided in Table 1. The PCR reaction mixture comprised of 10 µL of 2X AmpliTaq Gold PCR Master Mix (Applied Biosystems, Grand Island, NY, USA) and 1 µL each of forward and reverse primers (10 µM), with 1 µL of genomic DNA in the primary reactions. One µL of the amplicon from the primary PCR was used as a template in subsequent nested PCR assays, and sterile deionized water was added to achieve a final volume of 20 µL. Negative controls (reaction mixture without template DNA) were used in every set of PCR assays. Previously published protocols for *Rickettsia*-specific nested PCR primers and assay conditions were used to amplify the 23S-5S [20], and 17 kDa, and *gltA* [25] regions. For 23S-5S IGS PCR amplification, the first round of amplification conditions used were 95 °C for 10 min, 35 cycles of 94 °C for 30 s, 60 °C for 30 s, and 65 °C for 90 s, and a last cycle of 65 °C for 7 min. The second nested amplification conditions used were 95 °C for 10 min, 30 cycles of 94 °C for 30 s, 57 °C for 30 s, and 72 °C for 90 s, and a last cycle of 72 °C for 10 min. For the 17 kDa protein gene, conditions for the first round of amplification were 10 min at 95 °C, followed by 35 cycles of 95 °C for 30 s, 47 °C for 30 s, and 72 °C for 60 s, and a last cycle of 72 °C for 10 min. The nested PCR conditions were similar to those of the first round of PCR amplification, except that the annealing temperature was increased to 50 °C, and the cycles were repeated 30 times. The PCR conditions used for *gltA* were at 95 °C for 10 min, followed by 30 cycles of 94 °C for 30 s, 57 °C for 30 s, 72 °C for 60 s, and a last cycle of 72 °C for 5 min. The nested reaction included 95 °C for 10 min, 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 60 s, and a final cycle of 72 °C for 5 min. Two independent PCR assays were performed for each *Rickettsia* sp. for all three targets. The amplicons were subjected to electrophoresis on ethidium bromide-stained

1.2% agarose–Tris-acetate-EDTA gels. The amplicons were purified and subjected to Sanger sequencing using the forward primer from the nested reactions at Eton Bioscience, Inc. (Research Triangle Park, NC, USA).

Table 1. Nucleotide sequences of primers used in nested PCR.

Organism (References)	Primers	Sequence (5′ to 3′)	Amplification	Target Gene (Nested Amplification Product Size)
Rickettsia [20,21]	RCK/23-5-F	GATAGGTCRGRTGTGGAAGCAC	Primary	23S-5S target (~350 bp)
	RCK/23-5-R	TCGGGAYGGGATCGTGTGTTTC	Primary	
	RCK/23-5N1F	TGTGGAAG CACAGTAATGTGTG	Nested	
	RCK/23-5N1R	TCGTGTGTTTCACTCA TGCT	Nested	
Rickettsia [22]	17kD1_F	GCTCTTGCAACTTCTATGTT	Primary	17 kDa protein gene (232 bp)
	17kD2_R	CATTGTTCGTCAGGTTGGCG	Primary	
	17kN1_F	CATTACTTGGTTCTCAATTCGGT	Nested	
	17kN2_R	GTTTTATTAGTGGTTACGTAA	Nested	
Rickettsia [23,24]	RpCS.877p	GGGGGCCTGCTCACGGCGG	Primary	*gltA* (338 bp)
	RpCS1258n	ATTGCAAAAAGTACAGTGAACA	Primary	
	RpCS896p	GGCTAATGAAGCAGTGATAA	Nested	
	RpCS1233n	GCGACGGTATACCCATAGC	Nested	

2.6. Phylogenetic Analyses of Rickettsia Sequences

BLASTn and nucleotide sequence match analyses were used to compare partial nucleotide sequences of the 23S-5S IGS, 17 kDa, and *gltA* amplicons to those in the GenBank database. Sequences were aligned with the multiple alignment CLUSTALX software package [26]. Evolutionary distances between sequences of the 23S-5S, 17 kDa, and *gltA* amplicons and known *Rickettsia* species from the NCBI database were calculated (Kimura two-parameter model) [27], and phylogenetic trees were constructed separately for each region using the neighbor-joining (NJ) and maximum likelihood (ML) methods [28,29] from the MEGA X software package [30]. Bootstrap analyses, consisting of 1000 iterations, were performed to evaluate the robustness of tree topologies. All three *Rickettsia*-specific PCR targets were successfully amplified from chigger DNA samples C6, 12, 22, and 36, and single PCR targets were amplified from several additional samples. To further refine the taxonomic positions of these rickettsiae, sequences of the 23S-5S IGS, 17 kDa, and gltA genes were retrieved from the NCBI whole genome assemblies, and combined phylogenetic analyses were performed with concatenated sequences using MEGA X software. Evolutionary distances were calculated using the Kimura two-parameter model, and phylogenetic trees were inferred with NJ and ML analyses using MEGA X software, as described above.

3. Results

3.1. Chigger Infestation of Rodents

Chigger specimens were collected from two species of rodents at the two sites surveyed. The subsample of chiggers analyzed in the present study were removed from white-footed mice (*Peromyscus leucopus*), trapped in the South Mountains Game Lands in a typical hardwood forest habitat, and a cotton rat (*Sigmodon hispidus*) from Lake Norman State Park in a grassy field habitat near the edge of the forest. A total of 109 larval chigger mites of two different species were collected. The sequences submitted for BLASTn analyses showed 85 to 91% identity with homologous sequences from Trombiculidae species for the COI gene (GenBank accession number MG314759). Based on their morphology, chiggers collected from *P. leucopus* at South Mountains Game Lands were identified as

Leptotrombidium peromysci, and those collected from *S. hispidus* at Lake Norman were in the genus *Eutrombicula* (Table 2).

Table 2. Molecular detection of genus *Rickettsia* spp. in chigger mites.

Location *	Chigger-Infested Rodent Species	Species of Chigger	Number of Chiggers Screened	Number of Chiggers Positive for the Gene Fragment		
				23S-5S #	17 kDa #	*gltA* #
LNSP	*Sigmodon hispidus*	*Eutrombicula* spp.	17 (1)	9	4	3
SMGL	*Peromyscus leucopus*	*Leptotrombidium peromysci*	29 (4)	13	8	4
Total			46	22	12	7

* LNSP—Lake Norman State Park, SMGL—South Mountains Game Lands. # Samples were considered positive for genus *Rickettsia* spp. only when DNA of the expected size was amplified for a specific PCR target in both replicate assays. Numbers in parentheses indicate the number of individual rodent species from which chiggers were collected.

3.2. Rickettsia Infection Patterns

Amplification details of the three gene/23S-5S IGS targets for detection of *Rickettsia* spp. in chiggers are listed in Table 1. Twenty-two of the 46 chiggers we tested (47.8%) were positive for *Rickettsia* using the 23S-5S gene. Of those, we sequenced all of the 23S-5S amplicons and compared them by BLASTn to similar sequences in GenBank. Of the 22 amplicons, five sequences (C6, 12, 22, 47, 48) were 99.5–100% homologous with *R. felis* (NC007109). One amplicon (C8) had a 98.6% identity with *R. conorii* (NC003103), two amplicons (C7 and C33) had an 86.9% identity with *R. amblyommatis* (CP015012), and one amplicon (C36) had a 93.3% identity with *R. typhi* (NC017066). The remaining thirteen amplicons had ambiguous sequences (likely indicating more than one species of *Rickettsia* present).

The NJ phylogenetic tree based on 23S-5S IGS from *Rickettsia* amplicon sequences revealed that the *Rickettsia* detected in the chiggers were closely related to *R. felis*, *R. conorii*, *R. typhi*, and unidentified *Rickettsia* species (Figure 2). Similar results were obtained using the ML method (Supplementary Figure S1).

From the twelve 17 kDa amplicons, five had a 99.1 to 100% identity with *R. felis* (NC007109), and one had a 99.3% identity with *R. amblyommatis* (CP015012). Six of the amplicons had ambiguous sequences. NJ-based phylogenetic analyses of the 17 kDa gene sequences showed that *Rickettsia* spp. in chiggers were closely related to *R. felis* and *R. amblyommatis* (Supplementary Figure S2). These same relationships were also reflected in the ML tree (Supplementary Figure S3). Of the seven *gltA* amplicons, five showed 99 to 99.8% similarity with published sequences for *R. felis*, one sequence showed 99.8% similarity to *R. amblyommatis*, and one amplicon had ambiguous sequences. NJ-based phylogenetic analyses based on five *gltA* gene sequences (C6, C12, C22, C36, and C48) were closely related to *R. felis*, one sequence (C7) was closely related to *R. amblyommatis* (Supplementary Figure S4); the same relationships were also reflected in the ML tree (Supplementary Figure S5). Only four samples (C6, 12, 22, and 36) produced amplicons for all three PCR targets. The phylogenetic analysis with concatenated sequences, 23S-5S IGS, 17 kDa, and *gltA*, showed that chigger amplicon sequences C12 and 22 were clustered in the same clade with *R. felis*, C6 was sister to the *R. felis* clade, and C36 to the *R. typhi* clade (Figure 3). Similar results were found using the ML method (Supplementary Figure S6).

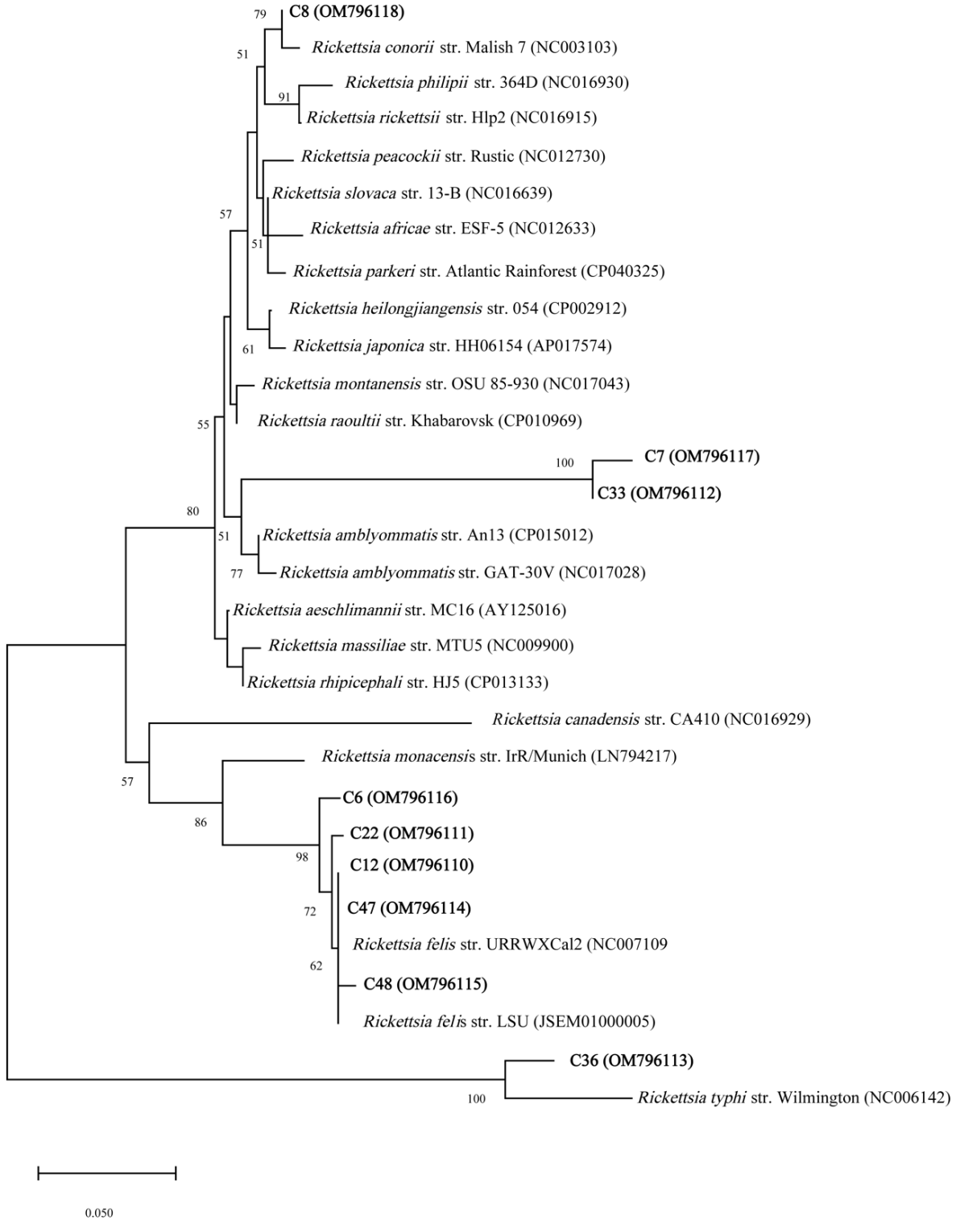


Figure 2. Phylogenetic relationships derived from partial sequences (~231 bp) of the *Rickettsia* 23S-5S IGS amplified from chigger DNA samples (highlighted in bold) and other related *Rickettsia* taxa, inferred using the NJ method. The GenBank accession numbers are given in the parentheses. Scale bars indicate the number of substitutions per nucleotide position.

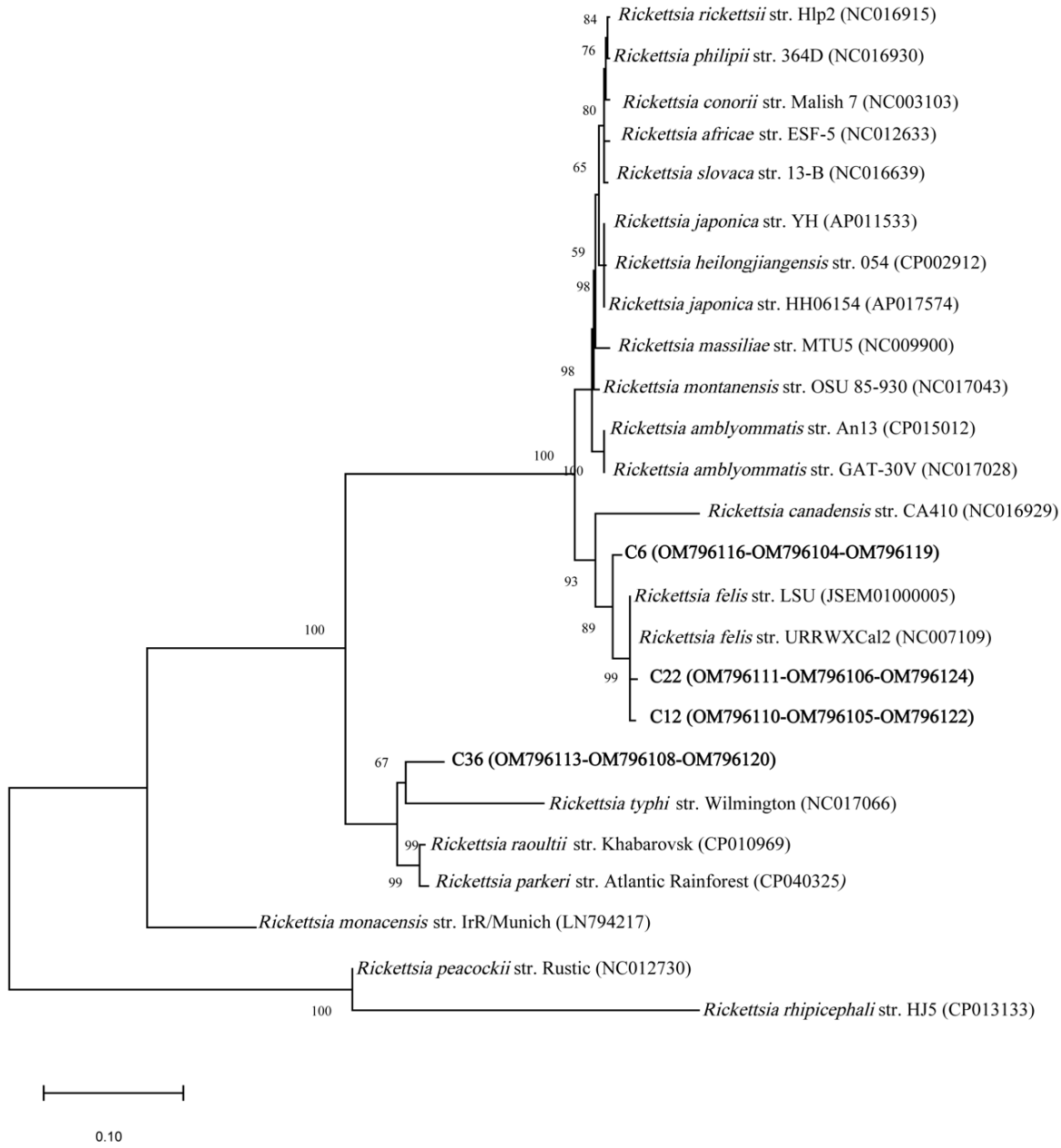


Figure 3. Phylogenetic tree of *Rickettsia* sequences from chiggers (bold letters) collected from rodents in North Carolina, USA, and reference sequences in GenBank (NCBI). The tree was inferred from the concatenated sequences (~733 bp) of three regions (23S-5S IGS, 17 kDa, and *gltA*) of the *Rickettsia* spp. genome by the neighbor-joining (NJ) method. The GenBank accession numbers are given in parentheses.

4. Discussion

In this study, we provide the first evidence of *Rickettsia* infection in chiggers collected from rodents within North America, a significant extension of recent work finding rickettsial DNA in trombiculid mites from South America and Asia [10,31,32]. Nucleotide sequence analyses of *Rickettsia*-specific 23S-5S IGS, 17 kDa, and *gltA* gene fragments indicated the amplicons from these chiggers were closely related to those in *R. felis*, *R. conorii*, *R. typhi*, and unidentified *Rickettsia* species. The most frequently detected rickettsial species in our study, *Rickettsia felis*, is globally distributed and was found in fleas, mosquitoes, ticks, and chigger mites [31]. There is increasing evidence that chiggers could be vectors of transitional group rickettsiae, because of their discovery in a variety of field-collected

chiggers [10,32]. Several other rickettsiae known to infect humans, namely *R. japonica*, *R. akari*, *R. felis*, *R. conorii*, *R. typhi*, and other *Rickettsia* spp. closely related to TwKM02 (*R. australis* and Cf15) were detected by molecular methods in chiggers collected from wild rodents in Korea [8]. Interestingly, chiggers in the US feed on the same rodent species that serve as blood meal hosts for ticks known to carry rickettsial species that cause spotted fever-type diseases, further raising the question of whether some infections attributed to ticks could in fact be derived from chiggers.

Historically, chigger mites were collected from rodents captured in the region of the 'Tsutsugamushi Triangle', in areas of Korea, Japan, throughout southern Asia, the Asian–Pacific region, and northern Australia [33,34]. Scrub typhus was thought to be exclusive to the so-called 'Tsutsugamushi Triangle', ranging from Pakistan in the west, far-eastern Russia in the east, to northern Australia in the south. However, recent scrub typhus reports in the Middle East, southern Chile, and Africa have reshaped this line of thinking, suggesting a wider geographical distribution of the disease [3,4,6,35–38]. The vectors of scrub typhus caused by *Orientia tsutsugamushi* (closely related to the genus *Rickettsia*, i.e., Rickettsiales: *Rickettsiaceae*) [1,2] are chiggers in the genus *Leptotrombidium* in the Tsutsugamushi Triangle [33,34], and in the genus *Herpetacarus* in Chile [2,6]. Both *Leptotrombidium* and *Eutrombicula* collected from rodents in our study in the US (known to bite humans, causing skin inflammation and hypersensitivity lesions [39,40]) were found infected with *Rickettsia* species. Additional epidemiologic studies are needed to examine whether there is a link between these rickettsiae-infected mites on wild rodents and human disease. An examination of *Orientia* species in US chiggers is also needed.

Based on morphological identification, two trombiculid genera, *Leptotrombidium* and *Eutrombicula,* were found on rodents in our study. However, our COI sequence data from these chiggers had no significant match with these genera. The BLAST search only matched with Trombiculidae. The lower sequence identity with the latter could be due to the lack of sequence information for US chiggers in GenBank. Presently, molecular data on chiggers in GenBank are mostly from southeast Asian and South American countries. Clearly, there are concerns about the present state of systematics of US chiggers that will require a significant level of future research effort to resolve (and which is outside the scope of this paper). Our study is the first to identify the genus *Rickettsia* in US chigger mites from rodents. Future studies to determine rickettsial infection in other ectoparasites on the same host, in hosts tissues, and in free-living chiggers, in order to understand possible vertical, transovarial transmission of rickettsiae would be informative.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/microorganisms10071342/s1. Figure S1. Phylogenetic relationships derived from partial sequences (~231 bp) of the *Rickettsia* 23S-5S IGS amplified from chigger DNA samples (highlighted in bold) and other related *Rickettsia* taxa. The phylogenetic tree was reconstructed using the maximum likelihood method. The GenBank accession numbers are given in the parentheses. Scale bars indicate the number of substitutions per nucleotide position; Figure S2. Phylogenetic relationships derived from partial sequences (~215 bp) of the *Rickettsia* 17 kDa gene amplified from chigger DNA samples (highlighted in bold) and other related *Rickettsia* taxa. The phylogenetic tree was reconstructed using the neighbor-joining method. The GenBank accession numbers are given in the parentheses. Scale bars indicate the number of substitutions per nucleotide position; Figure S3. Phylogenetic relationships derived from partial sequences (~215 bp) of the *Rickettsia* 17 kDa gene amplified from chigger DNA samples (highlighted in bold) and other related *Rickettsia* taxa. The phylogenetic tree was reconstructed using the maximum likelihood method. The GenBank accession numbers are given in the parentheses. Scale bars indicate the number of substitutions per nucleotide position; Figure S4. Phylogenetic relationships derived from partial sequences (~287 bp) of the *Rickettsia* citrate synthase (*gltA*) gene amplified from chigger DNA samples (highlighted in bold) and other related *Rickettsia* taxa. The phylogenetic tree was reconstructed using the neighbor-joining method. The GenBank accession numbers are given in the parentheses. Scale bars indicate the number of substitutions per nucleotide position; Figure S5. Phylogenetic relationships derived from partial sequences (~287 bp) of the *Rickettsia* citrate synthase (*gltA*) gene amplified from chigger DNA samples

(highlighted in bold) and other related *Rickettsia* taxa. The phylogenetic tree was reconstructed using the maximum likelihood method. The GenBank accession numbers are given in the parentheses. Scale bars indicate the number of substitutions per nucleotide position; Figure S6. Phylogenetic tree of *Rickettsia* sequences from chiggers (bold letters) collected from rodents in North Carolina, USA, and reference sequences in GenBank (NCBI). The tree was inferred from the concatenated sequences (~733 bp) of three regions (23S-5S IGS, 17 kDa, and *gltA*) of the *Rickettsia* spp. genome by the maximum likelihood method. GenBank accession numbers are given in parentheses.

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Biomolecular Minerals and Volcanic Glass Bio-Mimics to Control Adult Sand Flies, the Vector of Human *Leishmania* Protozoan Parasites

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Abstract: Sand flies (Diptera: Psychodidae) serve as vectors for transmitting protozoan parasites, *Leishmania* spp., that cause the disease called leishmaniasis. The main approach to controlling sand flies is the use of chemical insecticides. The discovery of alternative methods for their control is needed because of potential health risks of chemical insecticides and development of sand fly resistance to these pesticides. The biomineral produced by diatoms (diatomaceous earth, DE; Celite) and a volcanic glass bio-mimic (Imergard) have been shown by our group to be efficacious against mosquitoes, filth flies, and ticks but never studied for the control of sand flies. In a modified World Health Organization cone test, 50% of adult *Phlebotomus papatasi* sand flies at 29 ± 1 °C, 55 ± 5% RH, and 12:12 LD, when exposed to Imergard and Celite, were dead in 13.08 and 7.57 h, respectively. Proof of concept was established for the use of these biominerals for sand fly and leishmaniasis disease control. Using a light source as an attractant to the minerals had no significant effect on the LT_{50}, the time to 50% mortality. The LT_{50} at a higher relative humidity of 70 ± 5% increased to 20.91 and 20.56 h for Imergard and Celite, respectively, suggesting their mode of action was dehydration. Scanning electron microscopy of dead sand flies showed high coating levels of Celite only on the sides of the thorax and on the tarsi, suggesting an alternative mode of action for mechanical insecticides.

Keywords: industrial biomolecular minerals; sand flies; Imergard; Celite

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1. Introduction

Sand flies (Diptera: Psychodidae), mainly in the genus *Phlebotomus* and *Lutzomyia*, serve as vectors for transmitting protozoan parasites, *Leishmania* spp., in the family Trypanosomatidae, that cause the disease leishmaniasis [1]. The transmission of these parasites to humans occurs through the bite of an infected female sand fly. The three common forms of the disease are cutaneous, visceral, and mucocutaneous leishmaniasis [2]. Cutaneous leishmaniasis is characterized by the formation of skin lesions, which when numerous can cause permanent scarring and severe disability [3]. Visceral leishmaniasis affects multiple internal organs that can be fatal if untreated [4]. Mucocutaneous leishmaniasis results in partial or complete destruction of mucous membranes in the nasal, oral, and throat cavities and surrounding tissues [5]. Each form is species specific in the *Leishmania* genus. An estimated 1 billion people are at risk for contacting this disease, with annual incidence rates of 1 million cases of cutaneous leishmaniasis and 30,000 cases of visceral leishmaniasis [6].

The most common method used to control sand flies is chemical pesticide treatments, for example, insecticide-treated bed nets and indoor residual spraying [7,8]. However, the use of synthetic insecticides could have unintended consequences on non-target arthropods, could pose risks to human health, and would be less desirable to many people compared to a non-chemical, mechanical approach.

Diatomaceous earth (DE) is an inorganic biomolecule produced by diatoms, mined from earth deposits that have accumulated over eons of time. Some species like that in Celite 610 are insecticidal by a physical mode of action and have been used in insect management [9–11]. The application of DE to control vector important arthropods like sand flies is interesting because this approach has not been considered before and if successful could avoid the concerns associated with chemical pesticides. Also, the attempted development of other minerals that could mimic DE to control pest arthropods has largely been unsuccessful. Recently, we discovered a new mechanical insecticide called Imergard™ that is derived from volcanic rock as a DE mimic that was efficacious against mosquitoes [12,13], filth flies [14], and ticks [15], but was not examined against sand flies. The proposed mechanism of action for Celite and Imergard involves the abrasion of the insect's outer, cuticular layer through physical contact and/or the absorption of cuticular lipids, leading to dehydration and death [16].

The current study investigated the effectiveness of Celite and Imergard for the control of adult sand flies, *Phlebotomus papatasi*, under different environmental conditions. Research was also conducted to better understand the mode of action of these mechanical insecticides on sand flies. The long-term goal is to demonstrate proof of concept that DE and the biomolecular mimic of DE, Imergard, could be a new, non-chemical approach to adult sand fly control.

2. Materials and Methods

2.1. Sand Fly and Mechanical Insecticides

Phlebotomus papatasi larvae and pupae (stages L3 and pupae *P. papatasi*, Turkey strain PPTK, NR-44000) were obtained from the Walter Reed Army Institute of Research, BEI Resources, NIAID, NIH (American Type Culture Collection, 10,801 University Blvd., Manassas, VA, USA) and maintained through the adult stage under Level II biological containment (ACL-2, arthropod) in the Dearstyne Entomology Laboratory (3230 Ligon Street, North Carolina State University, Raleigh, NC, USA) by methods approved by NCSU Environmental Health and Public Safety (BUA #2019-10-818). Larval pots were placed in a plexiglass (30.48 × 30.48 × 30.48 cm) mosquito rearing cage [17], housed in a foldable butterfly rearing cage (Restcloud, Chengdu, China) in a walk-in incubator (maintained at 26 °C, 80% relative humidity, and 12:12 (L:D) cycle). The incubator was in a room in the Dearstyne Entomology Building, where the only access door was nominally closed and where the door opening into the room was covered with mosquito netting. Fly paper (two traps, Black Flag, distributed by the Chemsico Division of the United Industries Corporation, St. Louis, MO, USA) was deployed from the ceiling to kill any flies that might escape from the walk-in incubator. Access to the room with the walk-in incubator was restricted to only BSL II trained researchers, and the room was fitted with two Black Flag fly paper traps and a Model BZ-15N Black Flag Bug Zapper. No flies were ever detected outside of the walk-in incubator. The pots were opened twice a week to release adult flies into the mosquito-rearing cage (the first containment level outside of the pots). The pots were gently tapped to promote fly emergence from the soil substrate. The bottoms of the pots were soaked in 2.5 cm of glass-distilled water for 10 min each time to maintain an adequate moisture level. The mosquito cage contained a 50 mL plastic test tube held with the open end up, filled with a 30% sucrose solution in glass-distilled water and with a cotton wick projecting from the sucrose solution through the open end of the tube to provide food and water to the adult sand flies.

Mechanical insecticides, Imergard and Celite 610, were provided by the company Imerys (Imerys Filtration Minerals, Inc., Roswell, GA, USA). Imergard is made from volcanic rock and Celite is made from diatomaceous earth. Both products were maintained at room temperature and humidity in the dark in their original commercial packaging until used.

2.2. Bioassay

A modified WHO cone test device (Figure 1) was used to measure Imergard and Celite lethal activity against adult sand flies (mixed sexes). The device consists of a Petri dish bottom (13.8 cm diameter), glass funnel (with a 9.9 cm diameter large opening, a 1.6 cm diameter small opening, and a 12 cm height), orange stopper, and plastic cap [14]. Imergard or Celite was spread as evenly as possible on each petri dish bottom (Figure 1) using a clean metal spatula at the rate of 26.25 g/m^2. Twenty 5–10 d old (after emergence) adult flies (mixed sexes) were then transferred by aspiration from the mosquito cage to the cone test device, where the cap was removed and the orange stopper lowered to the middle of the funnel. After transferring, the orange stopper was raised to the sealed position and the cap re-applied. The cone test devices were then placed on a flat surface in an incubator at 29 ± 1 °C, 55 ± 5% RH, and a 12:12 L:D photoperiod. The bioassay modification from the WHO cone test [18,19] included the use of the stopper (Figure 1, not in the WHO test) and the assay bottom positioned on a flat surface (versus at an angle to the flat surface in the WHO bioassay method). Control tests were not treated with mechanical insecticides and were conducted at the same time as the treatments. Four replicates were conducted on different days. Flies were considered dead when there was no fly movement when gently moving the cone test device back and forth on the horizontal surface supporting the Petri dish bottom. Mortality data were recorded every three hours. The tests were started during the early part of the photophase, and in some cases, mortality had to be assessed during the scotophase by briefly shining a light source into the cone test.

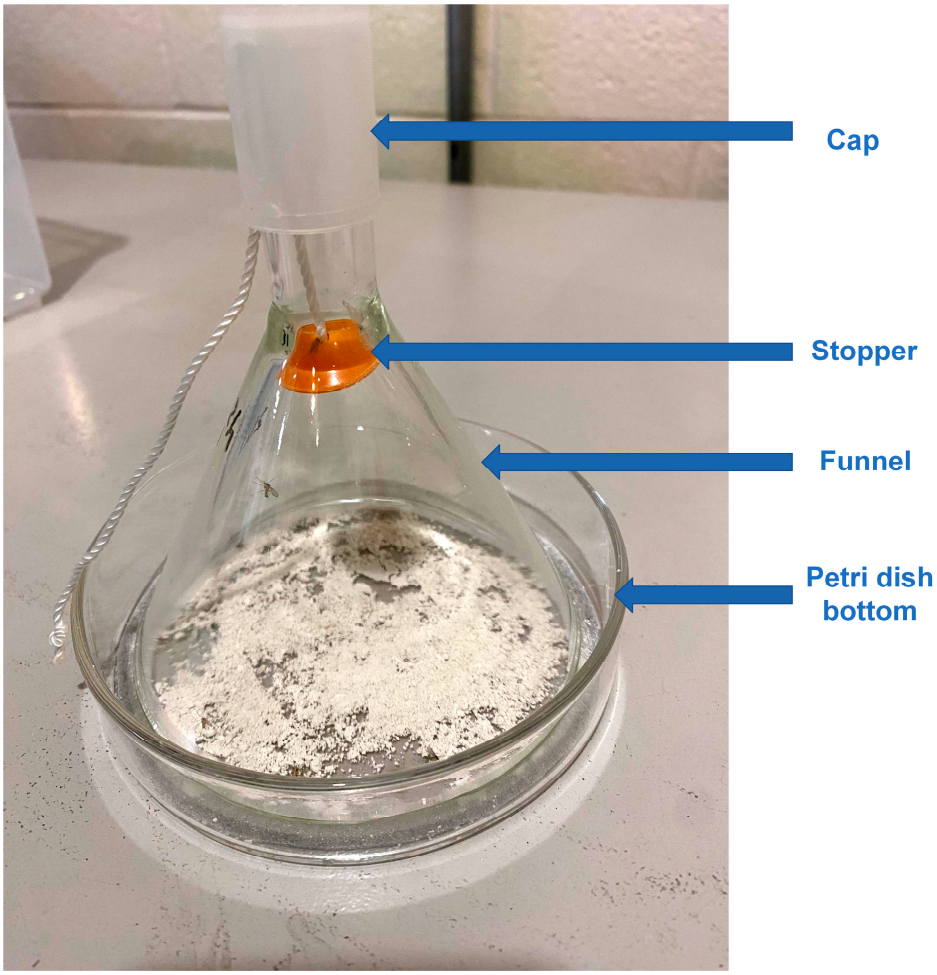


Figure 1. Architecture for the WHO modified cone test consisting of a cap, an orange stopper with a string, a funnel, and a Petri dish bottom. The white powder in the Petri dish bottom is Celite.

2.3. Bioassay with Light Source under the Petri Dish

In the bioassay described in Section 2.2, we observed the adult sand flies were standing mostly on the inside surface of the cone. This was not the case for similar studies conducted with mosquitoes [12] and with three species of filth flies [14]. Studies so far also suggested that mosquitoes, filth flies, and ticks were not significantly repelled by Celite or Imergard [12–15]. *P. papatasi* is known to be attracted to white light [20]. Therefore, we placed a light (Nebo Eye Smart Sensor Light, 260 Lumens; Washington, DC, USA) just below the Petri dish bottom to possibly increase sand fly interactions with Celite and Imergard. There was no increase in temperature from the use of the light. Twenty 5–10 d old adult flies were transferred as described earlier. The cone test devices were transferred to the incubator at 29 ± 1 °C, 55 ± 5% RH, and with the lights off in the incubator. Three replicates were conducted on different days. Control tests were not treated with mechanical insecticides and were conducted at the same time as the treatments. Observations were made as described in Section 2.2.

2.4. Bioassay at High Humidity

If dehydration is the mode of action of Celite and Imergard, increasing the humidity should increase the time to death. Twenty 5–10 d old adult flies were transferred as described earlier to each cone test device treated with Imergard or Celite (with no light source below the Petri dish). The cone test devices were then incubated at 29 ± 1 °C, 70 ± 5% RH, and a photoperiod of 12:12 (L:D). This experiment was repeated four times on different days. Control tests were not treated with mechanical insecticides and were conducted at the same time as the treatments.

2.5. Statistical Analysis

Time course data were analyzed by Probit analysis using SAS (Version 9.4, Cary, NC, USA) with mortality Abbott corrected [21]. The LT_{50} and LT_{80} are the times to 50% and 80% mortality, respectively, and were calculated using the Probit model with Abbott correction. When the 95% confidence limits of the LT_{50} and LT_{80} values do not overlap in comparisons, they are considered statistically significantly different.

2.6. Scanning Microscopy

To identify if mechanical insecticides had transferred from the treated surface to the sand flies in the modified cone test device, scanning electron microscopy (SEM) was used to visualize Celite on the surface of adult sand flies that had died in the cone test. Dead sand flies were transferred individually from the cone test bottom to a glass vial and sent to the Analytical Instrumentation Facility at North Carolina State University. After being vacuum dried for 48 h, sand flies were attached to an aluminum Hitachi SEM mount using super glue. After that, they were coated with a 70 nm gold-palladium mixture (60 Au/40 Pd) using Cressington sputter coating for 60 s. To capture images of Celite on the surface of the sand flies, they were examined using a Hitachi SU3900 (Hitachi, Ltd., Chiyoda City, Tokyo, Japan) variable pressure scanning electron microscope.

3. Results

3.1. Efficacy of Imergard and Celite against Sand Flies

In cone tests (Figure 1), adult sand fly (mixed sexes) mortality was first observed after 3 h of exposure to Celite and Imergard at 29 ± 1 °C, 55% RH, and a 12:12 L:D cycle (Figure 2). Percentage mortality reached 100, 93.75, and 18.75 percent at 30 h for Celite, Imergard, and the control, respectively, after which the test ended. A probit model for time versus Abbot corrected mortality was developed (Table 1) for the results in Figure 2 and used to calculate the time to 50% (LT_{50}) and 80% (LT_{80}) mortality. The LT_{50} for Imergard was longer at 13.08 h (12.00–14.21 h, the 95% confidence interval (CI)) than that for Celite (7.57 h, 6.87–8.25 h) (Table 1). The LT_{80} for Imergard was also longer (26.50 h, 23.77–30.31 h,

95% CI) as compared to Celite (13.30 h, 12.28–14.49 h). The LT_{80} was also significantly longer than the LT_{50} for both minerals tested (Table 1).

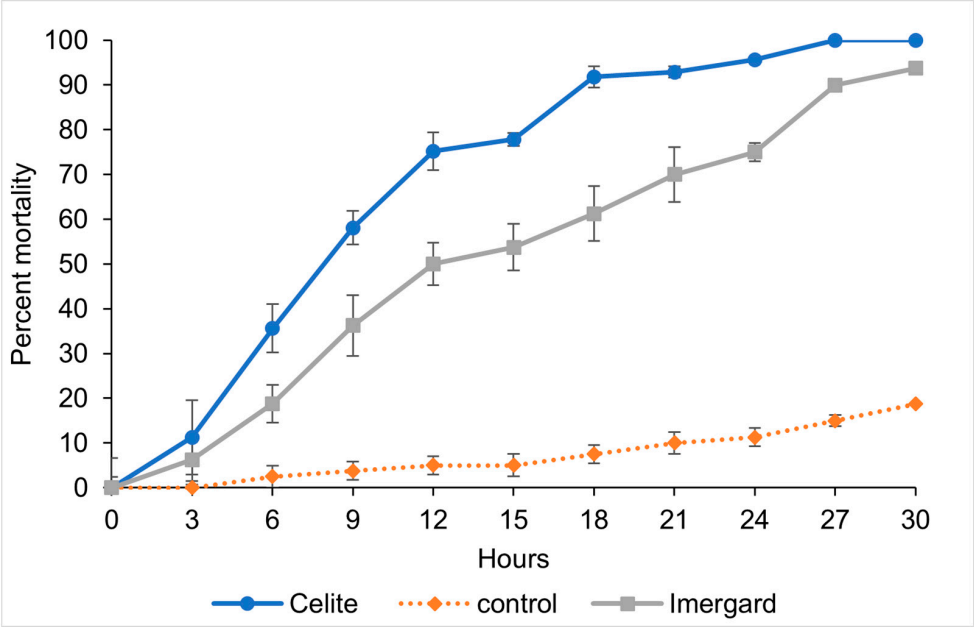


Figure 2. Time versus mortality for Imergard and Celite versus a negative control in a modified WHO cone test (Figure 1) at 29 ± 1 °C, 55 ± 5% RH and 12:12 L:D against mixed sexes of adult sand flies, *Phlebotomus papatasi*. The error bars are ±1 standard error of the mean, which in some cases were smaller than the size of the symbol.

Table 1. Probit model and time to 50% (LT_{50}) and 80% (LT_{80}) mortality of adult sand flies, *Phlebotomus papatasi*, in a WHO modified cone test.

Mineral	Environment	*n*	Slope (SE) [+]	LT_{50} (95% CL) [++]	LT_{80} (95% CL) [++]	χ^2 [+++]
Imergard	29 ± 1 °C, 55% RH	80	2.75 (0.20)	13.08Aa (12.00–14.21)	26.50Ab (23.77–30.31)	43.65
Celite	29 ± 1 °C, 55% RH	80	3.44 (0.22)	7.57Ba (6.87–8.25)	13.30Bb (12.28–14.49)	44.35
Imergard	29 ± 1 °C, 55% RH, light	80	2.17 (0.58)	11.59ABa (7.06–16.46)	28.34ACDb (19.31–75.89)	205.64
Celite	29 ± 1 °C, 55% RH, light	80	2.65 (0.30)	8.23Ba (6.72–9.66)	17.11Cb (14.63–20.80)	51.62
Imergard	29 ± 1 °C, 70% RH	80	1.81 (0.23)	22.16C (18.92–27.34)	N/A	53.90
Celite	29 ± 1 °C, 70% RH	80	4.32 (1.13)	20.82Ca (16.93–27.81)	32.59ADa (25.25–64.80)	418.40

[+] SE = standard error. [++] Results in hours, Abbott corrected. CL = confidence limit. In each column, different upper-case letters indicate no overlap of the 95% confidence intervals and were statistically significantly different. In each row, different lower-case letters indicated no overlap of the 95% confidence intervals and were statistically significantly different. [+++] Chi-square.

3.2. Effect of a Light Source on Sand Fly Mortality

A light source was placed underneath the Petri dishes in our modified WHO cone test, and the assays conducted with the overhead incubator lights off in an attempt to better attract the sand flies to Imergard and Celite on the inside surface of the Petri dish.

Mortality was first observed at 3 h for both Imergard and Celite at 29 ± 1 °C and 55 ± 5% RH (Figure 3). Percentage mortality reached 95, 88.3, and 18.3 percent at 30 h for Celite, Imergard, and the control, respectively, after which the test ended. The LT_{50} and LT_{80} for flies exposed to Imergard with the light source were 11.59 h (7.06–16.46 h, 95% CI) and 28.34 h (19.31–75.89 h, 95% CI), respectively (Table 1). There were no significant differences

in the LT_{50} and LT_{80} between assays with and without the light source. When exposed to Celite with the added light source, the LT_{50} was 8.23 h (6.72–9.66 h, 95% CI), and the LT_{80} was 17.11 h (14.63–20.80 h, 95% CI). At the LT_{50}, again, there was no difference with the addition of the light source between Imergard and Celite while at the LT_{80}, the time to 80% mortality was longer with the light source compared with no light source (Table 1). While there was a statistically higher time to 80% mortality for both Imergard and Celite compared to the LT_{50}, no differences were found between these minerals for the same end point, unlike what was found in the absence of the light source.

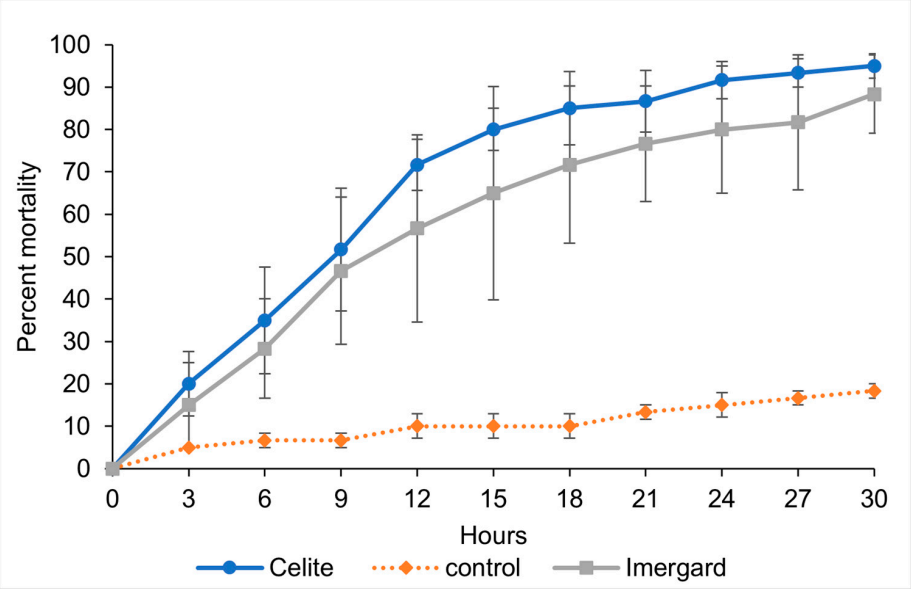


Figure 3. Time versus mortality for Imergard and Celite versus a negative control in a modified WHO cone test (Figure 1) at 29 ± 1 °C and 55 ± 5% RH (with the overhead incubator lights off and a light source below the glass Petri dish) against mixed sexes of adult sand flies, *Phlebotomus papatasi*. The error bars are ±1 standard error of the mean, which in some cases were smaller than the size of the symbol.

3.3. Effect of Humidity on Sand Fly Mortality against Minerals

Assays were also run under high humidity (29 ± 1 °C, 70 ± 5% RH and 12:12 L:D). Mortality first occurred at 3 h for Imergard and at 9 h for Celite, and the mortalities were 67.5 and 90%, respectively, at 30 h, after which the study was ended (Figure 4) and were similar to the ending times for studies at the lower humidity (Figure 2). The LT_{50} for flies exposed to Imergard at 70% RH was 22.16 h (18.92–27.34 h, 95% CI) (Table 1). The LT_{50} for Celite was 20.82 h (16.93–27.81 h, 95% CI). There was no significant difference between Imergard versus Celite treatments under high humidity conditions at the LT_{50} (Table 1). The LT_{80} for Celite was 32.59 h (25.25–64.80 h, 95% CI). We were not able to calculate the LT_{80} for Imergard without extrapolation because the percent morality did not exceed 80% at 30 h. The LT_{50} for both Imergard and Celite was higher at 70% compared to 55% RH (Table 1).

3.4. Scanning Electron Microscopy

Scanning electron microscopy was conducted on dead sand flies after their exposure to Celite (Figures 5 and 6). Three replicates were conducted, and the results shown are typical of all replicates. Only Celite was investigated because a US EPA registration for insect control is already granted, because of economy of time and resources, and because of limited access to instrumentation. The micrographs show Celite on the sand fly head, tarsi, wing, thorax, and abdomen. The amount of Celite in different parts of the sand flies varied. By far the highest coating levels were on the thorax (especially on the pleuron) and tarsi.

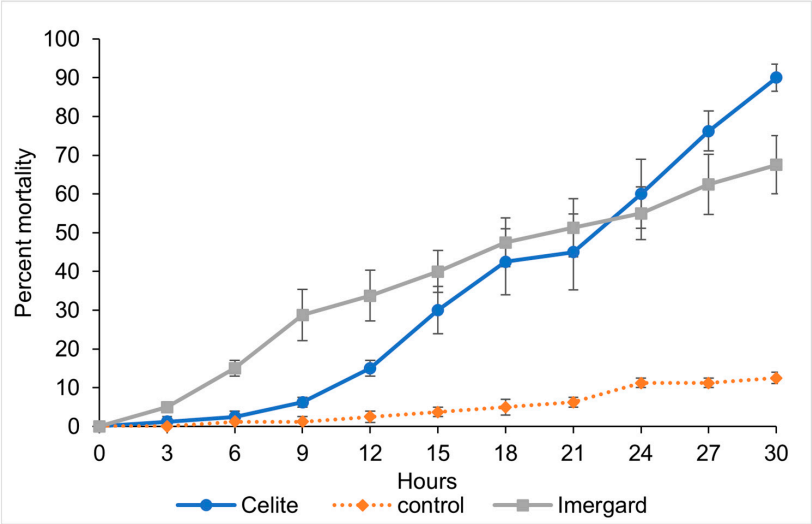


Figure 4. Time versus mortality for Imergard and Celite versus a negative control in a modified WHO cone test (Figure 1) at 29 ± 1 °C, 70 ± 5% RH, and a 12:12 L:D cycle against mixed sexes of adult sand flies, *Phlebotomus papatasi*. The error bars are ±1 standard error of the mean, which in some cases were smaller than the size of the symbol.

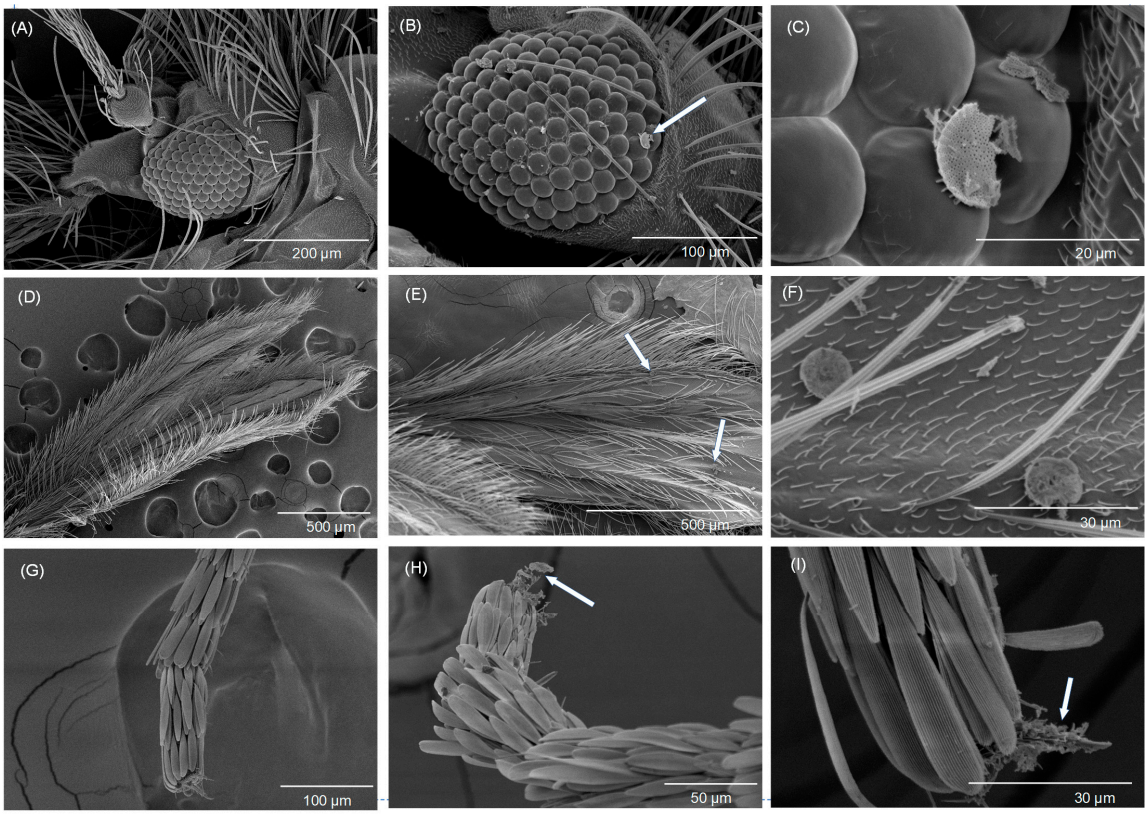


Figure 5. Scanning electron micrographs of an adult sand fly, *Phlebotomus papatasi*, exposed to Celite at the treatment rate of 26.25 g/m^2. Cones were placed in an incubator at 29 ± 1 °C, 55 ± 5% RH, and 12:12 L:D. Dead flies were transferred to glass vials individually for imaging. (**A**) untreated head, (**B**) Celite treated head, (**C**) Celite treated head—higher magnification, (**D**) untreated wing, (**E**) Celite treated wing, (**F**) Celite treated wing—higher magnification, (**G**) untreated lower leg, (**H**) Celite treated lower leg, and (**I**) Celite treated lower leg—higher magnification. Arrows are pointing to Celite on the fly cuticle, which is shown at a higher magnification in the picture to the right.

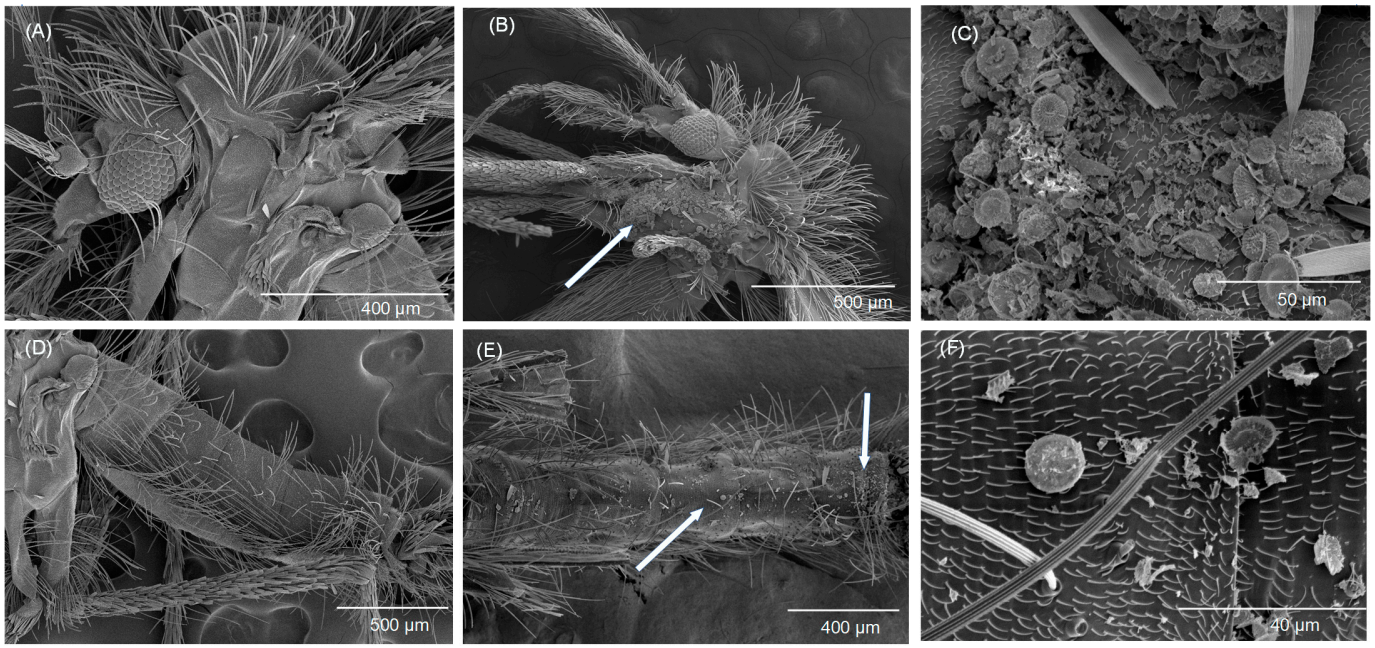


Figure 6. Scanning electron micrographs of an adult sand fly, *Phlebotomus papatasi*, exposed to Celite at the treatment rate of 26.25 g/m^2 in the cone test. Cones were placed in an incubator at 29 ± 1 °C, 55 ± 5% RH, and 12:12 L:D. Dead flies were transferred to glass vials individually for imaging. (**A**) untreated thorax, (**B**) Celite treated thorax, (**C**) Celite treated thorax—higher magnification of pleuron of the thorax, (**D**) untreated abdomen, (**E**) Celite treated abdomen, and (**F**) Celite treated abdomen—higher magnification. Arrows are pointing to Celite on the fly cuticle, which is shown at a higher magnification in the picture to the right.

4. Discussion

Two industrial minerals, diatomaceous earth (DE, Celite 610) made by diatoms and a volcanic glass bio-mimic Imergard developed for mosquito and malaria control in Africa, were investigated for their insecticidal activity against unfed adult (mixed sexes) of sand flies, *Phlebotomus papatasi*. Phlebotomine sand flies are vectors of *Leishmania* protozoan parasites that cause human leishmaniasis, one of the most significant vector-borne diseases in the world. The insecticidal properties of minerals produced by diatoms, such as the species found in Celite, were known before. However, its use in controlling sand flies was never studied. In addition, we developed a mimic to DE called Imergard for mosquito control, which was more efficacious than Celite [12]. Imergard is derived from volcanic glass and is easily mixed in water and applied with conventional spray equipment used in vector control; as a residual wall spray inside of homes in Africa, Imergard was efficacious by WHO standards, without significant loss of activity for 6 mo [12,13]. Imergard is abundantly available and is considered safe as the "white stuff found in toothpaste".

In a modified WHO cone test in this paper, we found that the LT_{50} for sand flies exposed to Imergard at 29 ± 1 °C and 55 ± 5% RH was 13.08 h; when exposed to Celite, the LT_{50} was 7.57 h. Deguenon et al. [12] used the same cone test and found that the Imergard LT_{50} for mosquitoes was 4.96 h at 32 ± 1 °C and 60 ± 5% RH. Chen et al. [14] found that the LT_{50}s for Imergard in the cone test for three species of filth flies were in the range of 3.2–7.1 h at 30 ± 1 °C and 50 ± 5% RH. Sand flies exposed to Imergard had a longer LT_{50} compared to mosquitoes and filth flies. However, our sand fly studies demonstrated similar susceptibility to DE with that of mosquitoes and filth flies.

In the filth fly studies conducted by Chen et al. [14], Imergard exhibited faster mortality rates against blow flies and house flies than against larger flesh flies. As insects increase in size (assuming similar body shapes), their volume increases cubically while their surface area increases by the square. Korunic [22] also reported that DE had a greater negative im-

pact on insects with smaller body sizes. This could be attributed to their larger surface area to volume ratio, leading to a comparatively higher loss of water compared to larger insects. The hypothesized mode of action of DE is disruption of the water protective layer of the insect cuticle by abrasion and/or absorption of lipids leading to dehydration and resulting in death. Sand flies in comparison to the filth flies studied by Chen et al. [14] are much smaller. Adult house flies weigh 20.1 mg [14] and adult sand flies weigh 0.22–0.30 mg [23]. The house fly adult is 67–91 times larger by weight than a sand fly. Based on size alone, the hypothesis would be that Imergard and Celite would have a greater effect on sand flies (have a shorter LT_{50}) than for the larger house fly. However, the opposite was discovered. *P. papatasi* are highly adapted to arid and periarid bioclimates with high temperatures and low relative humidity [24,25], and because of this adaptation they might have naturally occurring resistance mechanisms to prevent dehydration, which also increased their tolerance to Imergard. These possible resistance mechanisms that increased the time to the LT_{50} in sand flies are unknown.

The effectiveness of DE against insects was shown before by others to decrease when humidity increased [22]. At the higher humidity, the rate of water evaporation from the insect decreases. Chen et al. [14] also reported that the efficiency of Imergard decreased (the LT_{50} was longer) at a higher relative humidity for filth flies, and Deguenon et al. [12] found the same for malaria mosquito adults, *Anopheles gambiae*. In the current study, the LT_{50}s for Celite and Imergard against sand flies were longer as well at the higher humidity (Table 1), consistent with the hypothesized mode of action for these minerals which is dehydration. Increased humidity may also affect the electrostatic transfer of the mineral to the sand fly. There are no data on the impact of changes in humidity on electrostatics in the cone test.

Based on the scanning electron microscopy (SEM) images, Celite was predominantly observed on the pleural region of the thorax and on the tarsi of sand flies (Figures 5 and 6). There was mineral found on other areas of the body, but this was minimal by comparison. This is drastically different from what Chen et al. [14] found for house flies exposed to Imergard using the same modified WHO cone test used for sand fly exposures. The entire body of the house fly was coated with Imergard, and the fly appeared essentially as an "Imergard mummy". The setae on the pleuron of sand flies were much shorter with a lower density compared to that on the thorax dorsum, abdomen, wings, and head. This is different from house flies that have less setae of a shorter length. The functional reason for the long setae in sand flies is not completely clear but based on our findings with Celite (Figures 5 and 6), might be a mechanism to protect the sand fly from small sand particles and other debris (and from Celite) making contact to the body. This is further supported by the findings of Chen et al. [14] that house flies were heavily coated with Imergard while sand flies were not.

The sand fly coating results are informative, showing that mineral deposits mostly on the pleuron of the thorax and on the tarsi could have resulted in a lethal effect. The flies shown in Figures 5 and 6 were collected after they had died, while in the control experiments conducted at the same time with no minerals in the WHO funnel test, control mortality was zero. It is interesting that the spiracle that provides oxygen and carbon dioxide exchange to the brain and mouthparts is found (one each) on the pleuron of the thorax. Richardson et al. [15] hypothesized from SEM studies that nymphs of the black-legged tick, *Ixodes scapularis*, died after dipping into Celite because they had the spiracular plate blocked by the mineral, and Cave et al. [26] found the minerals inside of the aeropyles of the spiracular plate in adult *I. scapularis* that died by walking onto a treated surface of Celite. These studies suggested that the minerals might affect respiration or disrupt the water retention mechanisms associated with respiration. At the same time, the presence of minerals on the thoracic pleural region was correlated with mortality, but a "cause and effect" was not proven.

House flies in the modified WHO cone test [14] presented as much stronger fliers than the sand flies. House flies were more active and repeatedly flew at a high speed into the mineral-treated surface of the bioassay arena. Sand flies most often were seen standing on

the tapered side of the glass funnel. A light source under the assay arena was used in an attempt to enhance sand fly interactions with the treated Petri dish. Sand flies are known to be attracted to light [20]. However, no significant difference was observed on the time to the LT_{50} for both Imergard and Celite compared to assays conducted without light (Table 1). Richardson et al. [15] studied the efficacy of Imergard and Celite against unfed nymphal black-legged ticks and reported that Imergard exhibited slightly higher efficacy compared to Celite by dipping. In contrast, our findings indicated that sand flies exposed to Imergard had a longer LT_{50} compared to those exposed to Celite with no difference at high humidity (Table 1). Our results were different from the findings reported by Richardson et al. [15]. Factors that might increase the time to death for sand flies compared to the filth fly studies of Chen et al. [14] are differences in overall activity levels and flight dynamics, the ability for sand flies to rest on the glass sides of the funnel versus the treated bottom of the cone test, differences in insect electrostatics, and sand fly natural resistance to dehydration.

5. Conclusions

In conclusion, the biomolecular mineral produced by diatoms, Celite 610, and a volcanic glass mimic, Imergard, in a modified WHO cone test were lethal to adult sand flies, *Phlebotomus papatasi*, establishing as proof of concept that they could be used to control this pest and reduce human disease. Sand flies vector a protozoan that causes an important human disease, leishmaniasis. The time to 50% sand fly mortality was longer when exposed to Imergard and Celite at high relative humidity, consistent with the hypothesized mode of action for these minerals which is dehydration. Scanning electron microscopy of dead sand flies showed high coating levels of Celite found mostly on the sides of the thorax and on the tarsi, suggesting that water loss associated from the disruption of the outer cuticle water-proofing layer of the body in toto is not the mode of action. Alternatively, the minerals might be affecting the function of the water retention features of the respiratory system. Additional semi-field and field research is necessary to determine the practical use of Celite and Imergard in controlling sand flies.

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Comparative Efficacy and Mode of Action of the Bio-mineral Produced by Diatoms, Celite 610, against Three Species of Filth Flies

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Simple Summary: Filth flies like the house fly transmit on their body surface and internally microbes that cause disease in animals and humans. Use of chemical insecticides for fly control as given rise to resistance, and the need to develop alternate pest management tools. One approach is the use of bio-minerals from the skeleton of diatoms and volcanic glass mimics that mechanically cause insect dehydration and death. Proof of concept was demonstrated that the minerals from diatoms killed three species of filth flies, even under high humidity conditions, consistent with a dehydration mode of action. Scanning electron microscopy suggest the mechanism of dehydration is different from what is reported in the scientific literature.

Abstract: Flies are able to carry a wide range of microbes that cause diseases both on the outside and inside of their bodies. Chemical insecticides are used for fly control. Fly resistance to this chemistry is wide spread, and alternative, non-chemical approaches with a different mode of action are needed. We used a modified WHO cone test to determine the efficacy of the minerals produced by diatoms found in the commercial product, Celite 610, against house fly (*Musca domestica*), secondary screwworm (*Cochliomyia macellaria*), and grey flesh fly (*Sarcophaga bullata*) adults. Mortality was observed as early as 2 h with 50% and 90% mortality observed in as little as 3.5 and 4.6 h (the T_{50} and T_{90}) respectively, in house flies. The T_{50} and T_{90} increased as the size of the fly species increased (*M. domestica* to *C. Macellaria* to *S bullata*) and also increased for all three species when the relative humidity increased from 50 to 70% at 30˚C. These results suggest dehydration was the mode of action; however, scanning electron micrographs of live secondary screwworm adults 2 h after Celite exposure, showed the flies were evenly covered with the mineral with no obvious damage to the cuticle. Proof of concept was demonstrated that these minerals can be used for filth fly control and is discussed in the context of our volcanic glass bio-mimics developed for mosquito control.

Keywords: filth flies, mechanical insecticides, pest control, Celite 610, health

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1. Introduction

Flies are pests often associated with animals, decaying biological material, and pestilence. We now know that this is due to their ability to carry pathogenic microorganisms. Some flies have evolved to live alongside humans, known as synanthropic or filth flies. They breed in animal and human excrement and in almost any kind of biological decaying material (Greenberg 1971, 1973, Graczyk et al. 2001, Mingchay et al. 2014, Onwugamba et al. 2018). Filth flies are a nuisance (Sukontason et al. 2006, Deguenon, Travanty, et al. 2019, Deguenon, Zhu, et al. 2019) but also carry and transfer to surfaces they contact, bacteria, viruses, fungi, and parasites (Graczyk et al. 2000, 2001, 2005, Khamesipour et al. 2018). The house fly, *Musca domestica* L. (Diptera: Muscidae), is perhaps the most well-known, physically transporting on the outside of their body to humans over a hundred pathogens

(Khamesipour et al. 2018), e.g., *Escherichia coli* (Szalanski et al. 2004) and *Salmonella spp.* (Pava-Ripoll et al. 2012). The other method of transporting these pathogens is internal followed by defecation and regurgitation. Secondary screwworms, *Cocliomya macellaria* F. (Diptera: Calliphoridae) transfer pathogens similar to the methods of *M. domestica* (Blazar et al. 2011). In addition, *C. macellaria* and the grey flesh fly, *Sarcophaga bullata* Parker (Diptera: Sarcophagidae) cause myiasis (James 1947, Hall 1991).

Current methods to control filth flies rely heavily on chemical insecticides, for example, pyrethroids, organophosphates and others (Khan et al. 2013, Mullen and Durden 2018). Flies are developing resistance to these compounds (Scott et al. 2013). Alternative, non-chemical methods for control are used such as cleaning and cultural practices, but this approach is not always feasible and is laborious. An alternative approach to chemistry of interest to our group has been applications in the material sciences, one approach being the study of mechanical insecticides (MI). MIs are minerals that have a physical mode of action resulting in insect death. These MIs have been shown to be effective against a wide range of pests such as mosquitoes (Deguenon et al. 2020, 2021), flies (Chen et al. 2021, Islam and Rahman 2021), bedbugs (Doggett and Geary 2008), beetles (le Patourel 1986, Arthur 2000, Showler 2002, Athanassiou et al. 2004, 2016), fleas (Korunić et al. 2016), cockroaches (Faulde et al. 2006, Hosseini et al. 2014), thrips (Mitchell et al. 2018), and ticks (Showler et al. 2020, Richardson et al. 2022, Showler and Harlien 2023). The MI, Imergard™ WP, developed from volcanic glass was used as a residual wall spray and provided long lasting (six months) control of *Anopheles gambiae* Giles (Diptera: Culicidae) mosquitoes in Africa (Deguenon et al. 2020) and was shown to be efficacious under practical field conditions against other mosquito species in the US (Deguenon et al. 2021)

We have been studying the bio-minerals produced by diatoms known in general as diatomaceous earth (DE) and the specific species found in the commercial product Celite 610. Celite was effective as a mechanical insecticide against thrips in cotton (Mitchell et al. 2018), nymphal and adult ticks (Richardson et al. 2022, Cave et al. In review), and sand flies (Chen et al. In review) but was not studied before as a control method for filth flies. The advantage of Celite over that of Imergard, the former already has a US EPA pesticide registration. The purpose of this study is to determine proof of concept that the bio-minerals produced by diatoms found in Celite 610 could be used to control three common species of filth flies, *M. domestica, C. macellaria,* and *S. bullata*, and to investigate its mode of action. There is a common thinking that MIs are not efficacious under high humidity conditions.

2. Materials and Methods

2.1 Flies for Testing

House fly (*Musca domestica*), secondary screwworm (*Cocliomya macellaria*), and grey flesh fly (*Sarcophaga bullata*) adults were acquired from colonies reared at North Carolina State University (NCSU, Raleigh, NC, U.S.A.). *M. domestica* originally were obtained from Cornell University colonies started in 1982, a *C. macellaria* colony was established at NCSU from field collections in Raleigh, NC in 2012, and a *S. bullata* colony was started at NCSU from collections on the NCSU campus, Raleigh, NC in 2015. All flies were reared with a 16:8 (L:D) cycle, 25±2°C and 50% relative humidity. Adult flies of mixed sex and 8-15 d old were used for the studies that follow. *M. domestica* larvae were reared on a diet consisting of a homogenous mixture of 1:7.5:15 (v/v) of Calf-Manna (MannaPro®, Manna Pro Products, LLC, St. Louis, MO, U.S.A.), distilled water, and wheat bran (The Mennel Milling CO., Fostoria, OH, U.S.A.). *C. macellaria* and *S. bullata* larvae were reared on a 1.78:1 (v/v) mix of dry cat food (Purina Cat Chow Complete®, Nestlé Purina PetCare Company, St. Louis, MO, U.S.A.) to water. Dry food was added to the water 4 h prior to use.

2.2 Mechanical Insecticide and Cone Assay

The DE mechanical insecticide, Celite 610, was provided by the company, Imerys (Imerys Filtaration Minerals, Inc., Roswell, GA, U.S.A.) and was stored in its original packaging at room temperature and humidity until needed. A modified WHO cone test was utilized to determine fly mortality (Fig. 1). A glass funnel was placed, large opening down into the bottom of a glass Petri dish treated with Celite or no Celite for the control. The funnel has a diameter of 10 cm for the large opening and 1.6 cm for the small opening with a height of 12 cm. Flies inside of the cone (described later) were prevented from flying into the narrow neck of the funnel by an orange stopper held in place with a plastic cap that fits over the outside of the small neck of the funnel (Fig. 1). The Petri dish bottom was 13.8 cm in diameter and was treated with 5 g of Celite/m^2. One ml of the MI at 74.8 mg/mL in absolute ethanol (need to give purity, company, etc.) was added to the dish, and the dish tilted back and forth to cover the bottom of the dish in toto. The control was treated with 1mL of ethanol only. All dishes were placed flat on the bench top in a fume hood overnight to allow for complete evaporation of the ethanol. Flies were anesthetized with carbon dioxide gas at room temperature and pressure for 30 s, and 10 flies transferred with soft forceps to each Petri dish bottom after which they were covered by the capped funnel as previously described. Funnels and dishes were placed into a Percival I-36NL incubator (Percival Scientific, Inc, Perry Iowa, U.S.A.) at either 30˚C and 50% relative humidity or 30˚C and 70% relative humidity, both with a 16:8 L:D cycle. The number of dead flies were counted every half hour. Flies were counted as dead when they exhibited no movement after light taping of the funnel with a pencil. Three replicates of three cones per replicate with corresponding controls were performed each day and repeated on two additional days.



Figure 1. Example of the modified WHO cone test assay with Celite 610 present on the bottom

2.3 Scanning Electron Microscopy (SEM)

Live *C. macellaria* from one cone each (on the same day for the 50% relative humidity condition discussed earlier) for the treatment and control tests (2 h after the assay started) were anesthetized as described earlier and stored in separate groups (10 flies each) in separate 50 ml capped plastic centrifuge tubes (company name, etc.) at -40˚C until used. SEM was conducted at the Analytical Instrumentation Facility on the North Carolina State University Centennial Campus (Raleigh, NC, U.S.A.). The flies were desiccated in a vacuum for 48 h and then mounted with super glue (company, etc.) onto a Hitachi SEM aluminum mount (Hitachi, Ltd., Chiyoda City, Tokyo, Japan). The samples were then coated with a

Cressington sputter coating (company, etc.) for 60 s with a 70 nm gold–palladium mixture (60 Au/40 Pd). A Hitachi SU3900 (Hitachi, Ltd., Chiyoda City, Tokyo, Japan) variable pressure scanning electron microscope was used to image the fly surface.

2.4 Statistical Analysis

Mortality data were collated using Excel (Microsoft Co, Redmond WA, U.S.A.). Probit was performed in JMP Pro 16 (JMP Statistical Discovery LLC, Cary, NC, U.S.A.). Abbott's correction was utilized for the Probit models (Abbott 1987).

3. Results

3.1 Cone Assay Mortality

3.1.1. *M. domestica*

Fig. 2 is a SEM showing the composition of Celite used in our studies. Fig. 3 is the mortality of *M. domestica* flies exposed to this Celite over time in the modified WHO cone test (Fig. 1) during the photophase. The first mortality at 30±1˚C and 50±5% relative humidity for the Celite treatment was observed at 2 h with 89% mortality observed at 4.5 h. Control mortality under these same environmental conditions started at 2 h, but only reached 18% at 4.5 h (Fig. 3A). At 30±1˚C and 70±5% relative humidity, the first mortality occurred at 2.5 h, and 96% mortality was reached at 6.5 h (Fig. 3B). Control mortality under these same environmental conditions started at 2.5 h and reached 36% at 6.5 h (Fig. 3B). Neither data were Abbott corrected.

Mortality data were Abbott corrected for the Probit analysis (Table 1). The time to 50% mortality (LT_{50}) was 3.51 h and the time to 90% mortality (LT_{90}) was 4.61 h for the 30±1˚C and 50±5% relative humidity. The LT_{50} and LT_{90} for flies at 30±1˚C and 70±5% relative humidity was 4.11 h and 6.00 h, respectively. The LT_{50} and LT_{90} for each humidity were statistically significantly different (the 95% confidence intervals did not overlap).

3.1.2. *C. macellaria*

Results for the secondary screwworm adults exposed to Celite in the modified WHO cone test are shown in Fig. 4. Flies at 30±1˚C and 50±5% relative humidity began dying in the treatment at 2 h with 70% died at 4.5 h (Fig. 4A). Control mortality under these same environmental conditions started at 1.5 h, but only reached 11% at 4.5 h (Fig. 4A). At the same temperature with 70±5% relative humidity, the flies started dying in the treatment at 2.5 h and 96% were dead at 7.5h (Fig. 4B). Control mortality under these same environmental conditions started at 4.5 h, but only reached 11% at 4.5 h (Fig. 4B).

The timed mortality data were Abbott corrected and used to develop Probit models (Table 1). The *C. macellaria* LT_{50} was 4.10h and the LT_{90} was 5.25 h for the 30±1˚C and 50±5% relative humidity. At 30±1˚C and 70±5% relative humidity, the LT_{50} and LT_{90} were 4.84 h and 6.73 h, respectively. The LT_{50} and LT_{90} for each humidity were statistically significant different (no overlap of the 95% confidence intervals).

3.1.3. *S. bullata*

For the grey flesh fly, mortality at 30±1˚C and 50±5% relative humidity began in the treatment at 3 h with 97% dead at 11.5 h for (Fig. 5A). Control mortality under these same environmental conditions started at 6.5 h, but only reached 10% at 11.5 h (Fig. 3A). At 30±1˚C and 70±5% relative humidity, mortality began at 2.5 h with 97% achieved at 20 h (Fig. 5B). Control mortality under these same environmental conditions started at 6.5 h, and reached 32% at 20 h (Fig. 5B).

The Probit models with Abbot corrections is shown in Table 1 for *S. bullata*. The LT_{50} and LT_{90} for 30±1˚C and 50±5% relative humidity was 7.40 h and 10.2, respectively,

and the LT_{50} and LT_{90} for 30±1˚C and 70±5% relative humidity was 11.2 h and 16.7 h, respectively. The LT_{50} and LT_{90} for each humidity were statistically significantly different (no overlap of the 95% confidence intervals).

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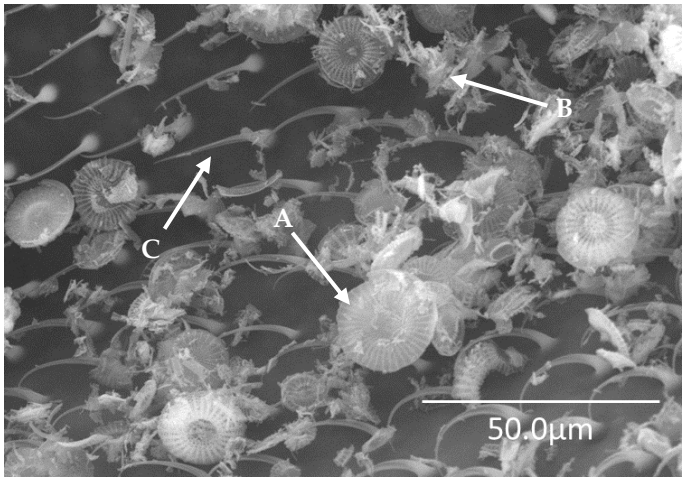
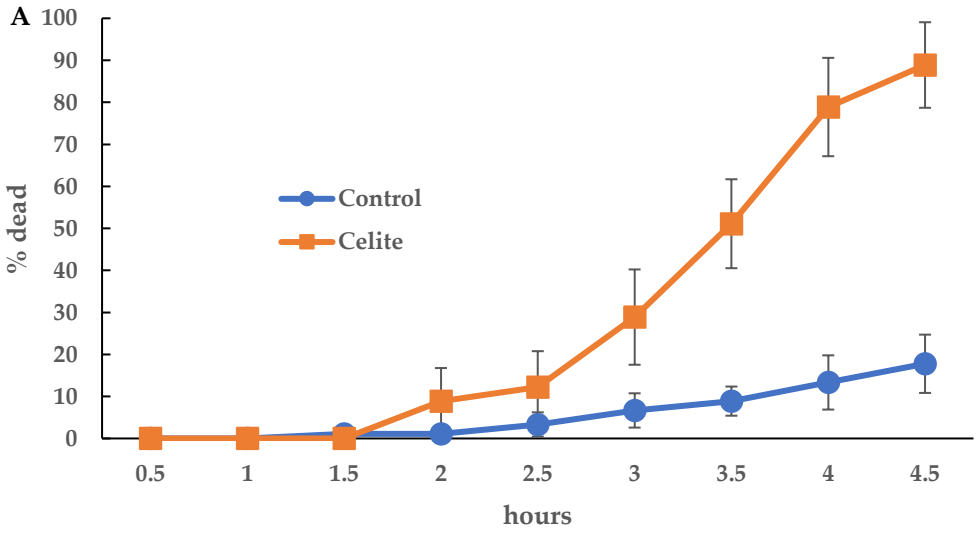


Figure 2. Scanning electron micrograph of the morphology of Celite 610 on the surface of the wing a secondary screwworm (*Cochliomyia macellaria*) fly. (A) An intact diatom. (B) A fragment of a diatom. (C) Setae on the surface of the wing.



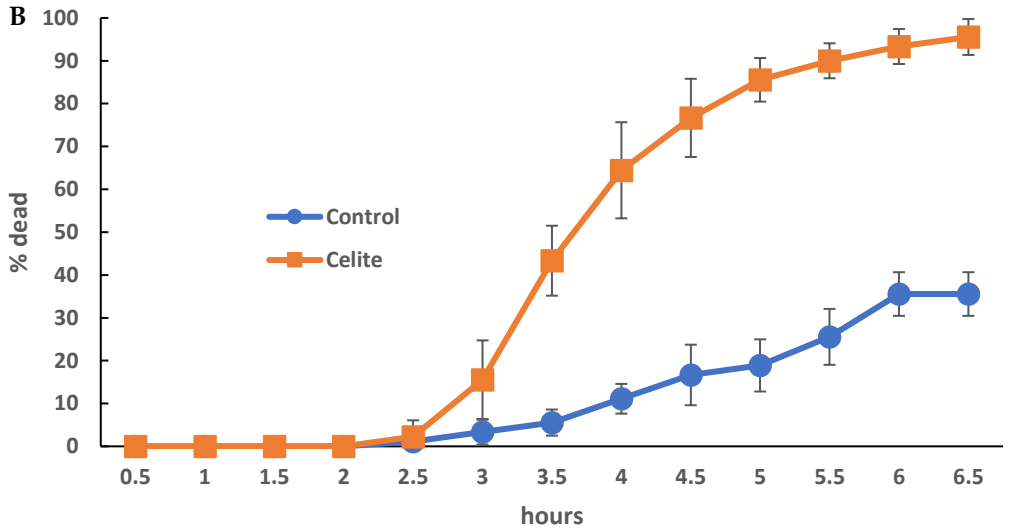


Figure 3. Mortality of unfed, mixed sex, adult house flies, *Musca domestica*, in the modified WHO cone test (Fig. 1) exposed to Celite (Fig. 2) at (A) 30±1˚C and 50±5% relative humidity and (B) 30±1˚C and 70±5% relative humidity (incubated during the photophase). Error bars are ±1 standard error of the mean. In some cases, the error bars were too small to be visible

Table 1. Probit models (Abbott corrected) for time (h) to 50 and 90% morlatity for three species of filth flies at 50 and 70% relative humidity when exposed to Celite 610 in a modified WHO cone test.†

Species	T/RH	N	Slope (SEM)	LT_{50}† (95% CL‡)	LT_{90}† (95% CL‡)	X^2
M. domestica	30C/50%	90	1.17(0.078)	3.51Aa (3.40-3.62)	4.61Ab (4.43-4.84)	85.0
	30C/70%	90	0.861(0.043)	4.11Ba (3.99-4.23)	6.00Bb (5.42-5.80)	68.9
C. macellaria	30C/50%	90	1.12 (0.0954)	4.10Ba (3.97-4.26)	5.25Bb (5.00-5.60)	83.2
	30C/70%	90	0.680(0.0317)	4.84Ca (4.70-4.98)	6.73Cb (6.52-6.97)	99.9
S. bullata	30C/50%	90	0.459(0.0173)	7.40Da (7.23-7.56)	10.2Db (9.93-10.5)	36.9
	30C/70%	90	0.233(0.00639)	11.2Ea (11.0-11.5)	16.7Eb (16.4-17.1)	140.4

†LT_{50} and LT_{90} is the time in h to 50 and 90% mortality, respectively (CL = confidence limit; X^2=chi-square).
‡Values with overlapping 95% CLs in the same column are considered not statistically significant and are designated by the same upper case letter. Lowercase case letters inform on statistically significant differences on the same row.

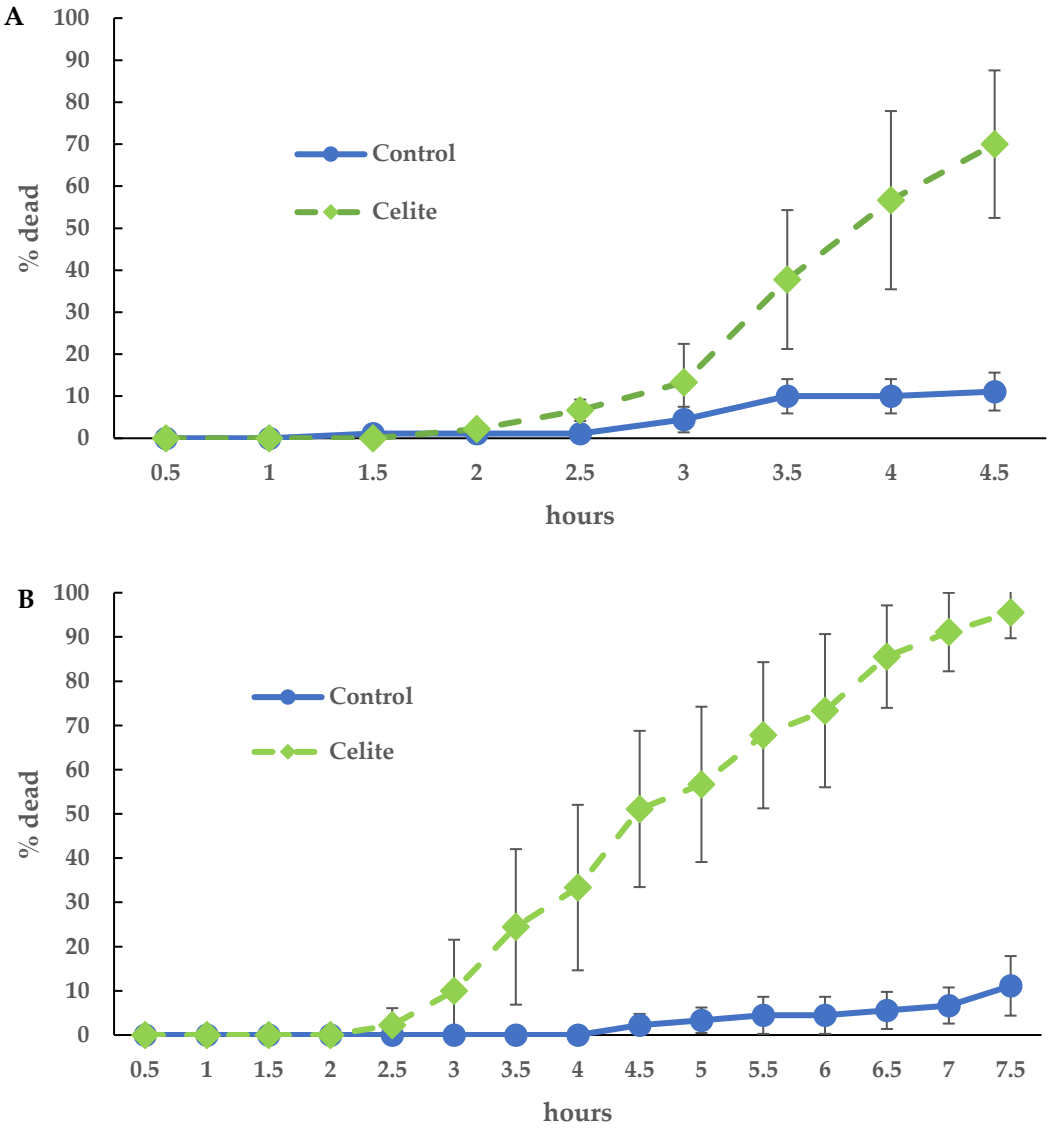


Figure 4. Mortality of unfed, mixed sex, secondary screwworm, *Cochliomyia macellaria*, adult flies in the modified WHO cone test (Fig. 1) exposed to Celite (Fig. 2) at (A) 30±1˚C and 50±5% relative humidity and (B) 30±1˚C and 70±5% relative humidity (incubated during the photophase). Error bars are ±1 standard error of the mean. In some cases, the error bars were too small to be visible.

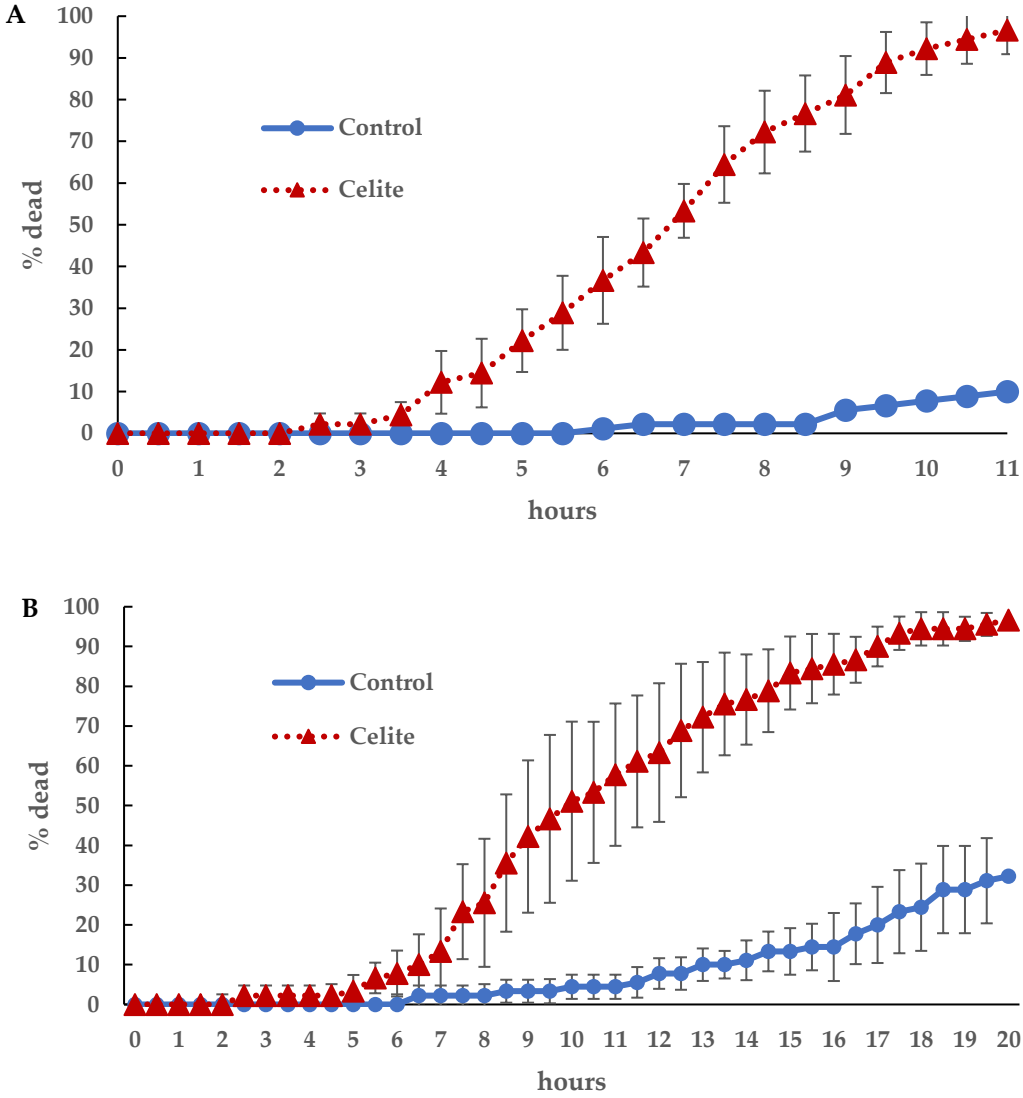


Figure 5. Mortality of unfed, mixed sex, grey flesh fly adults, *Sarcophaga bullata*, in the modified WHO cone test (Fig. 1) exposed to Celite (Fig. 2) at (A) 30±1˚C and 50±5% relative humidity and (B) 30±1˚C and 70±5% relative humidity. The incubation started in the photophase but the last 5h of observations were made during the scotophase. During the scotophase, the incubator lights were turned on briefly for the time needed to score mortality. Error bars are ±1 standard error of the mean. In some cases, the error bars were too small to be visible.

3.2 *Scanning electron microscopy of C. macellaria*

Fig. 4. is the scanning electron microscopy (SEM) of live *C. macellaria* exposed to Celite 610 versus the control with no Celite for 2 h at 30±1˚C and 50±5% relative humidity in the cone test (Fig. 1). Celite is extensively covering all parts of the insect. There is an even coating also on all parts. Fig. 2 shows a close up of the intact skeletons of the diatoms and skeletal fragments on the surface of the wing. There was no visible damage to the cuticle including setae due to the Celite (Fig. 2).

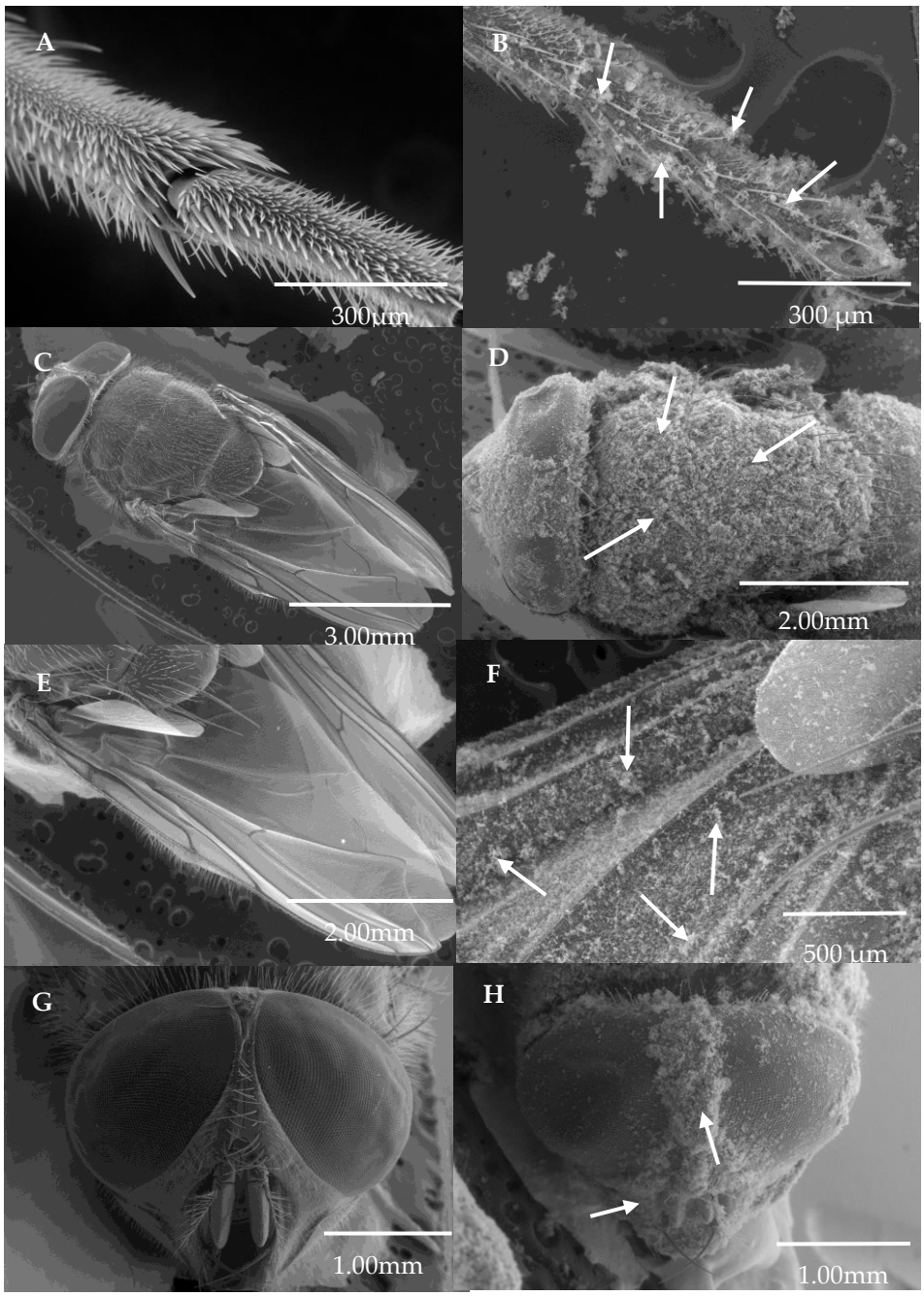


Figure 6. Scanning elecron microscopy of adult *C. macellara* control (left) and Celite exposed (right) from the cone tests. (A-B) leg, (C-D) body, (E-F) wing, and (G-H) head. Arrows are pointing to Celite.

4. Discussion

With the abilities of flies to vector disease causing pathogens, or cause health issues such as myiasis, there is much research into control of these pests. Currently there is an increase in pesticide resistance, so new methods of control are needed. The effect of Celite against filth flies had not been previously explored, and the concept was proven with this study. It has further been enhanced by Chen et al. who explored the the rate of death of flies exposed to Imergard. We found that flies exposed to Celite were similar to what was found, with death beginning at around 1.5-3 h at 30±1˚C and 50±5% relative humidity for all species. This in turn meant it could provide an applicable substitute for chemical insecticides. Furthermore, the comparisons show the efficacy of Imergard, and the LT_{50} and

LT_{90} of each of the species at 30±1˚C and 50±5% relative humidity was not statistically significant from the mortality of the Celite exposed filth flies here. *M. domestica*, for example, had a LT_{50} of 3.51 h and LT_{90} of 4.61 h when exposed to Celite and those exposed to Imergard was 3.2 h and 5.7 h respectively (Chen et al. 2021). The other species tested followed similar ranges against Imergard. This trend was also shown for other insects. Mosquitoes exposed to Imergard had a LT_{50} of 4.96 h at 32±1˚C and 60±5% relative humidity (Deguenon et al. 2021). The LT_{90} of the different species were also shown to differ from the LT_{50} of the same species at the same humidities. *S. bullata* had a LT_{50} of 7.40 h compared to the LT_{90} of 10.2 h. This change is only a 1.38 fold change, with the other species having a similar difference of 1.31 fold and 1.28 fold change for *M. domestica* and *C. macellaria* at 50% relative humidity respectively. This indicates that these mechanical insecticides provide control and can be used against filth flies. Not all conditions are at 50% relative humidity, pests also exist in tropical and subtropical conditions, therefore any pesticide would have to be able to function in such conditions. These results are comparable to other pests treated with Imergard and Celite. Richardson et al. found nymphal *I. scapularis* ticks had an LT_{50} of 1.36 h and 1.73 h against Celite and Imergard respectively. Showler et al. performed a similar test using *Amblyomma Americanum* (Ixodida: Ixodidae) larvae and nymphs and found that at or after 6 hours, it should be noted that LT_{50} was not calculated, but 100% mortality was found by 24 h. The efficacy of MIs have been tested against pests at higher humidities and found mortality to decrease (Korunić et al. 2016), and was shown in *An. Gambiae* (Deguenon et al. 2021) and for filth flies when exposed to Imergard (Chen et al. 2021). This trend continued with Celite and was found here. *S. bullata* displayed a LT_{50} of 11.2 h and LT_{90} of 16.7 h, a 1.49 fold change, at 70% relative humidity. *M. domestica* displayed a 1.46 fold change and *C. macellaria* was a 1.39 fold change. All of these results support the conceptual use of Celite. By comparison, the exposure of *An. gambiae*, by Deguenon et al. displayed a similar time to that found for *M.* domestica and *C. macellaria* despite a dramatic difference in sizes. has been hypothesized to be due to dehydration higher humidity would prevent some degree of water loss and thusly increate the time until mortality (Ebeling 1971, Mewis and Ulrichs 2001, Lilly et al. 2016, Malia et al. 2016). In addition, there was a trend of the larger species taking longer to die. This was earlier shown by Chen et al. with Imergard. The relative sizes of each fly differs, with *S. bullata* weighing 3 and 5 times that of *C. macellaria* and *M. domestica* respectively (Chen et al. 2021). As the size of the insect increase, the surface area to volume ratio also changes. In the case of *S. bullata* the ratio is smaller which means a larger mass and less surface area, and leads to less water loss (Kühsel et al. 2017). It should be noted that there is one exception to the trend of higher humidity and longer LTs, and this was found in nymphal ticks (Richardson et al. 2022). In the study Richardson et al. found that a higher humidity translated to faster death. This is hypothesized to be due to the ticks attempting to avoid dehydration by reducing activity in lower humidity environments (Vail and Smith 1998, Berger et al. 2014, Sonenshine and Roe 2014). With the data on dehydration, it should be noted that, as evidenced by the time in this study, Celite is still able to act as an insecticide at higher humidity. Further studies would need to be done to determine the effective range of humidity.

As stated above, it has been hypothesized that the dehydration and subsequent mortality is caused by two modes of action; removal of lipids, or abrasion of the insect cuticle. The SEM pictures (Fig 2, 6.) do not indicate any damage to the cuticle due to the Celite. This is supported by other research where death occurred for insects exposed to MIs. Ticks (Cave et al. to be published; Richardson et al. 2022), filth flies (Chen et al. 2021), mosquitoes (Deguenon et al. 2020), and sand flies (Chen et al. to be published), all did not display any damage due to MIs. The pests were all covered by the mechanical insecticide in each case. The only deviation of this is the greatest support for the lack of cuticular damage. The study performed by Chen et al. of the effects of Imergard against sand flies, found that the flies did die from exposure to Imergard, but the MI was only found on the side and tarsi of the insects (Chen et al. to be published). This lack of coverage is indicative of

the MI acting in a different function than as an abrasive or lipophile. SEM taken of the wing taken during this study support this conclusion (Fig. 2). The setae are whole, despite being covered by both whole and fragmented Celite.

The studies performed here have demonstrated that Celite is able to kill adults of three species of filth flies in the lab, but any pesticide will also have to function in field conditions. Celite has not been tested in field studies against filth flies at this point, but field studies with MIs have been performed on two other pests, tobacco thrips (*Frankliniella fusca* Hinds) and *An. gambiae*. In both studies the MI used, Celite against thrips and Imergard against *An. gambiae*, proved to be effective and able to control the pests. The study by Deguenon with *An. gambiae* even showed protection as a residual wall spray up to 6 mo before the study was halted. These two tests are encouraging for the use of MIs in the protection of agriculture and human health.

5. Conclusions

In conclusion, we demonstrated proof of concept that the bio-mineral produced by diatoms and found in Celite 610 was efficacious in killing house fly, secondary screwworm and grey flesh fly adults (mixed sexes). Time to mortality increased with an increase in relative humidity and decreased as the size of the fly decreased between species, consistent with dehyration as the mode of action. How the mineral causes dehydration was not clear from our studies.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Figure S1: title; Table S1: title; Video S1: title.

Author Contributions Conceptualization, G.L.C., K.C., and R.M.R.; methodology, G.L.C., K.C. and R.M.R.; software, G.L.C..; validation, G.L.C., K.C., and R.M.R.; formal analysis, G.L.C.; investigation, G.L.C.; resources, S.S.D., D.W.W., and R.M.R.; data curation, G.L.C.; writing—original draft preparation, G.L.C., and K.C.; writing—review and editing, G.L.C., K.C., and R.M.R.; visualization, G.L.C. and R.M.R.; supervision, R.M.R.; project administration, R.M.R.; funding acquisition, R.M.R. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: Data available upon justifiable request by the corresponding author.

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Conflicts of Interest: The authors declare there is no conflict of interest.

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Detection of *Orientia* spp. Bacteria in Field-Collected Free-Living *Eutrombicula* Chigger Mites, United States

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Scrub typhus, a rickettsial disease caused by *Orientia* spp., is transmitted by infected larval trombiculid mites (chiggers). We report the molecular detection of *Orientia* species in free-living *Eutrombicula* chiggers collected in an area in North Carolina, USA, to which spotted fever group rickettsiae infections are endemic.

Rickettsioses are distributed worldwide, caused by bacteria in the family Rickettsiaceae, genera *Orientia* and *Rickettsia* (*1*). The pathogens are transmitted by host-feeding arthropods, including ticks, mites, fleas, and lice (*2*). Among those arthropods are trombiculid mites, which have a widespread global distribution and high species diversity. Among the different stages of the lifecycle of trombiculid mites, only the larvae are ectoparasites, commonly known as chiggers (*3*). Some species are vectors of intracellular bacterial pathogens in the genus *Orientia* that causes a potentially lethal human febrile disease, scrub typhus (*4*,*5*). Scrub typhus results in considerable illness and death; it causes >1 million cases of illness each year (*5*). A recent review (*6*) concluded that trombiculid mites might be widespread vectors of other zoonotic agents not yet recognized. Spotted fever group rickettsiosis diseases, including Rocky Mountain spotted fever, have many of the same symptoms as scrub typhus. A recent review of the literature from 1997–2017 estimated >60% of the rickettsial diseases outside the United States were misdiagnosed (*7*).

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Until recently, scrub typhus was exclusively reported from the so-called tsutsugamushi triangle, stretching from Pakistan in the west to far-eastern Russia in the east to northern Australia in the south. However, scrub typhus was reported recently in the Middle East, southern Chile, and Africa (*5*,*8*). The occurrence of scrub typhus pathogens in chiggers in the United States was not investigated. We report the identification of *Orientia* species in free-living chiggers collected at recreational parks in North Carolina, USA.

The Study

In 2022, we collected free-living chiggers using the tile method (*9*) in different locations in North Carolina: we placed tiles on the ground and then visually inspected for the presence of chiggers after ≈1 minute. When chiggers were present, we collected them with forceps or a small paintbrush and transferred them into vials with 95% ethanol. We identified subsamples from each collection location based on morphological characteristics using published taxonomic keys (*10*). We identified chiggers as *Eutrombicula* on the basis of their morphology. In addition, we obtained chigger images of *Eutrombicula* using scanning electron microscopy at the Analytical Instrumentation Facility at North Carolina State University (Raleigh, North Carolina, USA) (Appendix Figure 1, https://wwwnc.cdc.gov/EID/article/29/8/23-0528-App1.pdf).

We surface-sterilized individual free-living chiggers and extracted total nucleic acids using the DNeasy Blood & Tissue Kit (QIAGEN, https://www.qiagen.com) (*11*). In total, 95 chiggers from 10 different locations (10 chiggers/location; 1 location had 5 chiggers) were subjected to microbiome analyses (Figure 1). We randomly selected 8 chiggers for molecular identification using previously described 18S ribosomal RNA gene primers and PCR (*12*). Amplicons were Sanger

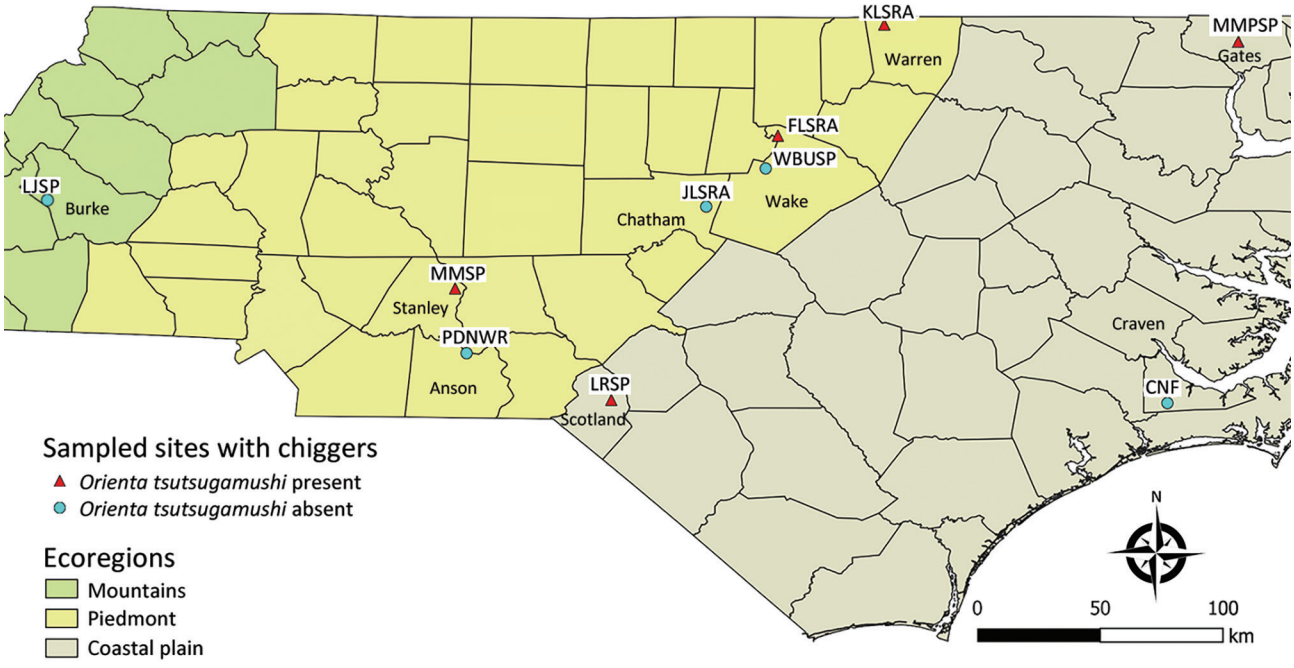


Figure 1. Study area for investigation of *Orientia* spp. bacteria in field-collected free-living *Eutrombicula* chigger mites, North Carolina, USA. Free-living chiggers were collected from 10 sites in 8 counties. CNF, Croatan National Forest; FLSRA, Falls Lake State Recreation Area; JLSRA, Jordan Lake State Recreation Area; KLSRA, Kerr Lake State Recreation Area; LJSP, Lake James State Park; LRSP, Lumber River State Park; MMPSP, Merchant Millpond State Park; MMSP, Morrow Mountain State Park; PDNWR, Pee Dee National Wildlife Refuge; WBUSP, William B. Umstead State Park.

sequenced at Eton Bioscience, Inc. (https://www.etonbio.com). The sequences (GenBank accession nos. OQ789321–5) were submitted for BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi) analysis and showed 99–100% identity with homologous sequences of *Eutrombicula* spp. (accession no. KY922159).

To determine the total bacteria present in the chiggers, we constructed a 16S rRNA sequence library of individual chiggers using the Illumina 16S rRNA (V3-V4 region) metagenomics sequencing library preparation protocol (Illumina, https://www.illumina.com) (*13*). Sequencing was performed at the University of North Carolina Microbiome Core Facility (Chapel Hill, North Carolina, USA). The Illumina FASTQ files were processed using Quantitative Insights into Microbial Ecology 2 (*14*) (Appendix). An analysis of amplicon sequence variants (ASVs) revealed that chigger mites contained reads of a bacterial sequence classified as *O. tsutsugamushi*. *O. tsutsugamushi*–positive chigger sequence reads were found in 5/10 sampling sites as follows: 3 sites in the Piedmont region (Falls Lake State Recreation Area [1 positive/10 chiggers], Kerr Lake State Recreation Area [8 positive/10 chiggers], and Morrow Mountain State Park [1 positive/10 chiggers]); and 2 sites in the Coastal Plains region (Lumber River State Park [9 positive/10 chiggers] and Merchant Millpond State Park [1 positive/5 chiggers]) (Figure 1). By using the Greengenes 16S rRNA database, we found 13 ASVs to *O. tsutsugamushi*. To further confirm those results, we extracted representative sequences from the sequencing data and conducted BLASTn searches against the National Center for Biotechnology Information (NCBI) databases. We found 12 ASVs showed a high nucleotide identity (99.5%–100%) to the *O. tsutsugamushi* strain Kato (D38624), isolated from a human scrub typhus case in Kurosawa village, Japan (https://u.osu.edu/scrubtyphus/the-kato-strain). One ASV exhibited 89.46% homology to an *O. tsutsugamushi* sequence that was excluded from the phylogenetic analysis. We then examined the approximate phylogenetic relationships for 12 of the *O. tsutsugamushi* sequence variants from our study sites with other *O. tsutsugamushi* sequences obtained from the NCBI database using BLASTn (accessed January 18, 2023). We performed multiple alignments with the ClustalW program and maximum-likelihood method tree with the Kimura 2-parameter method from the MEGA X (https://www.megasoftware.net) software package. The phylogenetic analysis revealed that all 12 of the *O. tsutsugamushi* ASVs clustered closely to *O. tsutsugamushi* strains from Asia (Figure 2).

To further verify the identity of *O. tsutsugamushi* detected in our free-living chigger samples, we amplified a 47-kDa *htrA* (high-temperature requirement A) gene in the 20 chigger samples that were positive for the *O. tsutsugamushi* 16S rRNA gene. The primers for the first round of PCR were *Ot*-145F and *Ot*-1780R, and for the second round, *Ot*-263F and *Ot*-1133R (*15*) (Appendix). The 47-kDa gene amplification products were Sanger sequenced at Eton Bioscience. Four samples (FC28, 36, 38, 109) were 92.6%–97.29% identical to the *O. tsutsugamushi* HN82 strain (GenBank accession no. LC431268) and 92%–97% identical to the *O. tsutsugamushi* Kato strain (accession no. LS398550) after trimming low-quality sequences. Sixteen samples yielded ambiguous sequences, suggesting the presence of multiple *Orientia* species or primer binding sites caused by high variation in the 47 kDa gene among *Orientia* species in our samples. Jiang et al. (*15*) studied the genetic variation of

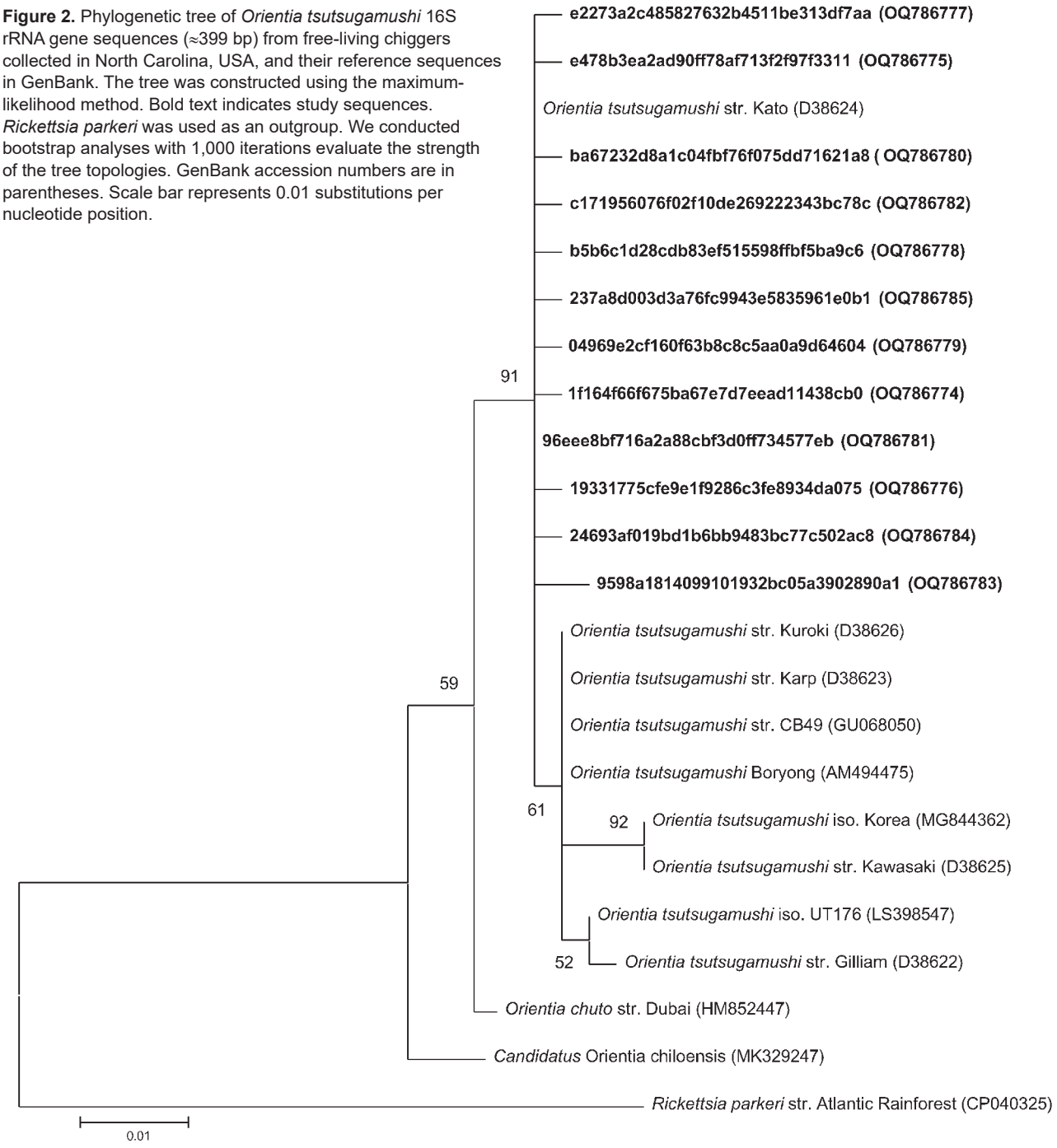


Figure 2. Phylogenetic tree of *Orientia tsutsugamushi* 16S rRNA gene sequences (≈399 bp) from free-living chiggers collected in North Carolina, USA, and their reference sequences in GenBank. The tree was constructed using the maximum-likelihood method. Bold text indicates study sequences. *Rickettsia parkeri* was used as an outgroup. We conducted bootstrap analyses with 1,000 iterations evaluate the strength of the tree topologies. GenBank accession numbers are in parentheses. Scale bar represents 0.01 substitutions per nucleotide position.

Orientia in this region of the genome and reported percent identity of 17 isolates of *Orientia* as 82.2%–83.3% (*15*). Among our 4 *Orientia* 47 kDa sequences from North Carolina chiggers, identity was 93.97%–98%. Phylogenetic analysis revealed that all 4 of those *O. tsutsugamushi* sequences clustered to *O. tsutsugamushi* strains from Asia (Appendix Figure 2).

Conclusions

This study identified *Orientia* species within the United States in free-living *Eutrombicula* chiggers that were collected in North Carolina. This result is epidemiologically significant because it indicates vertical circulation of *Orientia* species in chiggers collected within the continental United States. The presence of *Orientia* species in free-living larvae suggests that the bacteria are maintained through transovarial transmission. Further studies are needed to complete sequencing of the 47-kDa *htrA* gene (htrA) in our samples, determine how widely distributed *Orientia* spp.–infected free-living and host-attached chiggers are in the United States, and ascertain whether wild animals that serve as hosts for chiggers become infected and infectious and develop symptoms of illness. Clinicians in this region should be alert for possible human cases of illness resulting from *Orientia* spp. infection.

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Detection of *Orientia* spp. Bacteria in Field-Collected Free-Living *Eutrombicula* Chigger Mites, United States

Appendix

Bioinformatic Analysis of 16S rRNA Gene Sequencing Data from Free-Living Chiggers

The Illumina FASTQ files were processed using Quantitative Insights into Microbial Ecology 2 (QIIME2) (*1*). The DADA2 (*2*) plugin was used for removing noise and merging the paired-end reads, with any chimera removed. Primer sequences were trimmed, and low-quality sequences were removed by truncating the forward and reverse sequences at 280 and 220 nt, respectively. The DADA2 method replaces the traditional OTU-picking process, and instead models the sequence error to construct the actual biologic sequences present in the samples, referred to as Amplicon Sequence Variants (ASVs). The taxonomy classification was performed on the representative sequences generated from DADA2 using a Bayes classifier trained with Greengenes 13_8 at 99% identity (*3*).

Amplification of 47-kDa Gene of *Orientia* spp. from Chigger DNA

We amplified the 47 kDa gene of *Orientia* in 20 chigger samples that were positive for *Orientia* of 16S rRNA gene using nested PCR. The primers for the first round PCR were Ot-145F (5′-ACAGGCCAAGATATTGGAAG-3′) and Ot-1780R (5′-AATCGCCTTTAAACTAGATTTACTTATTA-3′). The primers for the second round PCR were Ot-263F (5′-GTGCTAAGAAARGATGATACTTC-3′)-and Ot-1133R (5′-ACATTTAACATACCACGACGAAT-3′) (*4*). In the first round of PCR mixture (10 μL) comprised 5 μL of reaction buffer, 1 μL each of forward and reverse primers at a concentration of 10 μM, 1 μL of genomic DNA, and 2 μL of sterile deionized water. For subsequent nested PCR assays (25 μL) comprised 1 μL of the amplicon from the first round of PCR was used after which 12.5 μL of reaction buffer, 1 μL each of forward and reverse primers at a concentration of

10 μM, and 9.5 μL sterile deionized water were added. Negative control reactions were included in each PCR run. The PCR amplification was performed using the following thermocycling conditions: initial denaturation at 95°C for 10 minutes, followed by 40 cycles of denaturation at 94°C for 30 seconds, annealing at 53°C for 30 seconds, and extension at 68°C for 2 minutes, with a final extension step at 72°C for 10 minutes, and a final hold at 4°C. The PCR products were subjected to Sanger sequencing at Eton Bioscience, Inc. (Research Triangle Park, NC, USA).

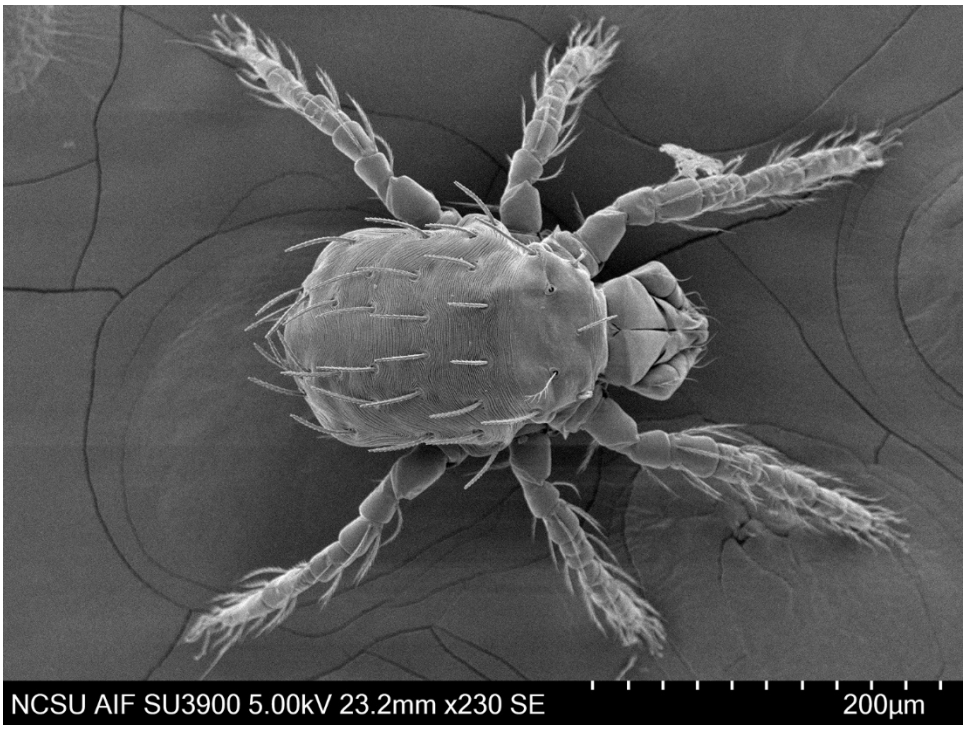
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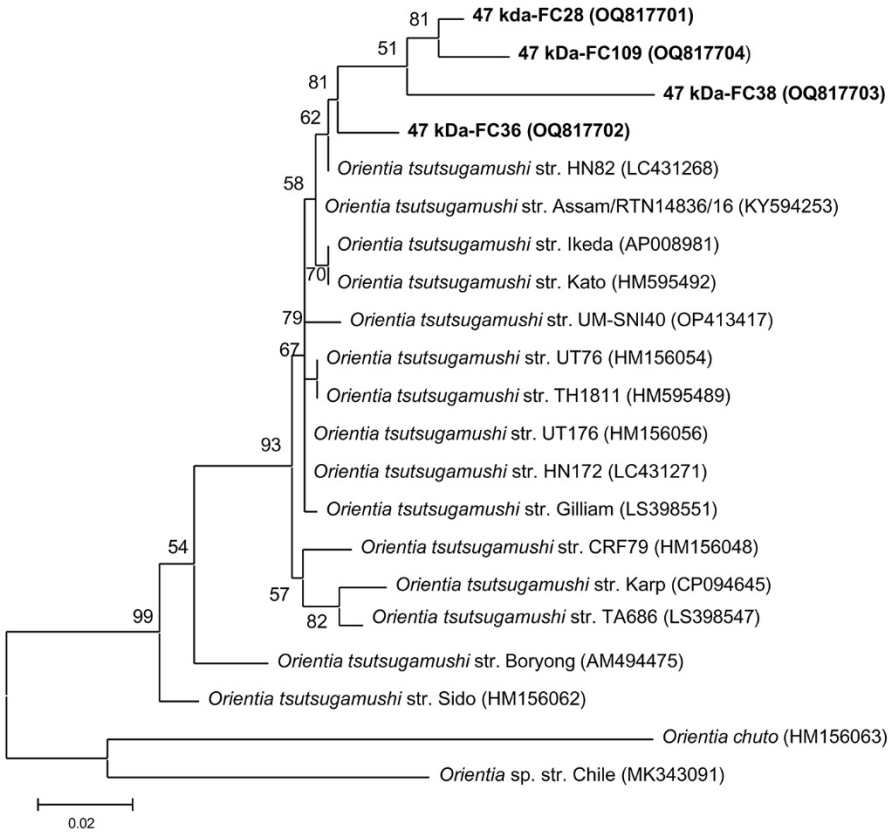
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Appendix Figure 1. Scanning electron micrograph of a *Eutrombicula* chigger.



Appendix Figure 2. Phylogenetic tree of *Orientia* spp. 47 kDa sequences (≈400 bp) from free-living chiggers collected in North Carolina, USA (bold text), and their reference sequences in GenBank (in parentheses). The tree was constructed using the maximum-likelihood method. To evaluate the strength of the tree topologies, bootstrap analyses were conducted with 1,000 iterations. Scale bar represents 0.02 substitutions per nucleotide position.

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Review

Rickettsia amblyommatis in Ticks: A Review of Distribution, Pathogenicity, and Diversity

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Abstract: *Rickettsia amblyommatis* is a potentially pathogenic species of *Rickettsia* within the spotted fever group vectored by ticks. While many studies have been published on this species, there is debate over its pathogenicity and the inhibitory role it plays in diagnosing illnesses caused by other spotted fever group *Rickettsia* species. Many publications have recorded the high infection prevalence of *R. amblyommatis* in tick populations at a global scale. While this species is rather ubiquitous, questions remain over the epidemiological importance of this possible human pathogen. With tick-borne diseases on the rise, understanding the exact role that *R. amblyommatis* plays as a pathogen and inhibitor of infection relative to other tick-borne pathogens will help public health efforts. The goal of this review was to compile the known literature on *R. amblyommatis*, review what we know about its geographic distribution, tick vectors, and pathogenicity, assess relatedness between various international strains from ticks by phylogenetic analysis and draw conclusions regarding future research needed.

Keywords: *Rickettsia amblyommatis*; *Amblyomma* spp.; *Amblyomma americanum*; lone star tick; tick-borne pathogens

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1. Medical and Veterinary Importance of Ticks

Ticks and the pathogens they vector represent a significant threat to humans, livestock, companion animals, and wildlife. Ticks are responsible for transmitting a greater variety of pathogens than any other arthropod group [1,2]. Cases of tick-borne disease have been increasing within the United States, having doubled from >22,000 cases in 2004 to >48,000 cases in 2016, with Lyme disease accounting for 82% of these reports [3]. According to the CDC, reported tick-borne disease cases increased to 50,865 in the USA in 2019 [4]. Some of the tick-borne diseases that impact people include Lyme disease (*Borrelia burgdorferi*), Rocky Mountain spotted fever (*Rickettsia rickettsii*), tick-borne encephalitis (*Flavivirus*), babesiosis (*Babesia microti*), anaplasmosis (*Anaplasma phagocytophilum*), ehrlichiosis (*Ehrlichia ewingii* and *Ehrlichia chaffeensis*), and others [5,6]. Some of the most important species of ticks that are responsible for the spread of important human pathogens include *Amblyomma americanum*, *Amblyomma maculatum*, *Dermacentor variabilis*, *Ixodes pacificus*, *Ixodes ricinus*, *Ixodes scapularis*, *Rhipicephalus sanguineus*, and others [5–7]. These tick-borne diseases can be life-threatening, and while some vaccines have ongoing clinical trials, vaccine availability is currently lacking [8–10]. Tick-borne diseases are responsible for significant economic losses within the livestock industry and present a threat to public health worldwide [2]. The incidence of cases of tick-borne diseases is projected to increase as climate change continues to alter environmental conditions that may change the distribution of tick species [1,11–13]. This may be especially true for *rickettsial* spotted fever illnesses with ticks as their primary vector. Due to the enhancement of cell culture systems and molecular techniques, the number of identified species of *Rickettsia* has increased significantly over the past 40 years [14–18].

2. Materials and Methods

To write this review, five electronic databases (Web of Science, Google Scholar, PubMed, ScienceDirect, and SciELO) were searched during the time frame of 1 May to 5 December 2022 for relevant publications spanning multiple decades. To the best of our knowledge, we have written a review that is representative of all of the databases investigated. For this search, the following keywords were used: "*Rickettsia amblyommatis*", "*Rickettsia amblyommii*", and "*Candidatus* Rickettsia amblyommii". The molecular phylogenetic analyses were completed using MEGA11 software (MEGA11: Molecular Evolutionary Genetics Analysis version 11) [19] using a maximum likelihood method on the Kimura 2-model (ML; bootstrap replicates: 1000). These blast searches were conducted on 3 February 2023.

3. Background on *Rickettsia*

The genus *Rickettsia* includes a diverse group of intracellular Gram-negative bacteria that are often the causative agents of disease for a variety of pathogens vectored by arthropods. Species within the genus of *Rickettsia* are split into four groups, including the spotted fever group (SFG), typhus group, *Rickettsia bellii* group, and the *Rickettsia canadensis* group [20–22]. It has also been recently shown that *Candidatus* Rickettsia mendelii is a separate basal group [23]. *Rickettsia* species are transmitted by a variety of arthropod vectors such as ticks, chiggers, fleas, and lice [24–27]. The maintenance of *Rickettsia* is dependent on animal hosts, mainly vertebrates; however, the role of these hosts in the life cycle of these bacteria is poorly understood [28]. Some hosts provide the necessary bloodmeal to ticks, and others provide essential supplemental components for the transmission of pathogens [28]. The spotted fever group (SFG) comprises the majority of tick-borne *Rickettsia*, consisting of 29 validly published species [29]. The primary reservoirs and vectors of SFG *rickettsia* are ticks. SFG *rickettsia* can move transstadially and transovarially, with some species of *Rickettsia* living within the entire life cycle of the tick [27,30]. Some species of *Rickettsia* are pathogenic and lethal causing serious morbidity in humans, while others are endosymbionts or symbionts not known to play an active role in human disease [30–32]. Some tick-borne *Rickettsia* species and the diseases they cause include *R. rickettsii*, *R. parkeri*, *R. akari* (spotted fever rickettsioses), *R. africae* (African tick bite fever), and *R. australis* (Australian tick typhus). In the United States, Rocky Mountain spotted fever (RMSF) caused by *R. rickettsii* is currently considered the most lethal identified rickettsial disease to humans [6,33,34]. Until 2010, most patients with tick-associated rickettsiosis were considered to have RMSF caused by *Rickettsia rickettsii* [35,36]. However, SFG rickettsial species elicit cross-reactive antibodies, and the immunofluorescence tests used by US public health labs cannot distinguish between the *Rickettsia* species that cause these different diseases [37]. Therefore, the CDC, as of 2010, now classifies cases formerly reported as RMSF as cases of SFG rickettsioses.

While rickettsioses are some of the oldest known vector-borne diseases, there is still much that is unknown and even debated, especially regarding each species' potential pathogenicity and status as being infectious or symbiotic [14,27]. With the arrival of molecular techniques, the understanding of *Rickettsia* has expanded. Still, there remain gaps in our knowledge, and with endemic rickettsioses cases on the rise, it is essential to understand these diseases fully. Over the last two decades, there has been great debate over the pathogenicity and significance of a specific ubiquitous species of *Rickettsia*, *Rickettsia amblyommatis*, which will be the focus of this review.

Background on Rickettsia amblyommatis

Rickettsia amblyommatis (formerly *Candidatus* Rickettsia amblyommii) was first discovered in Tennessee, USA in 1973 from the tick, *Amblyomma americanum* [38,39]. After this initial finding, *R. amblyommatis* was discovered infecting *A. americanum* populations at high percentages in South Carolina, USA, and Arkansas, USA [38]. Since then, *R. amblyommatis* was discovered infecting tick populations at high prevalence worldwide, reaching greater than 90% in the USA and Panama [40–42]. While *R. amblyommatis* has primarily been

isolated from ticks, it was recently discovered in chiggers (*Leptotrombidium peromysci* and *Eutrombicula* spp.) in North Carolina, USA [43]; however, this review will focus solely on ticks. *Rickettsia amblyommatis* was previously and informally described as *Rickettsia amblyommii* in the Stothard 1995 dissertation [44]. This species' name as well as *Candidatus* R. amblyommii was used throughout publications until 2016 when Karpathy et al. [38] formally assigned the name, *R. amblyommatis*, to conform to the rules of the International Code of Nomenclature.

Since its initial discovery, *R. amblyommatis* has proven to be an increasingly prevalent tick-borne rickettsial species, having been detected in 34 species of ticks in 17 countries (Figure 1; Table 1). Most significantly, *R. amblyommatis* has continually been detected at high prevalence in the USA in *A. americanum* populations, a geographically widespread human-biting tick [9,40,45–48]. In addition to being ubiquitous, *R. amblyommatis* is reported to have interesting other properties such as preventing ticks from acquiring other coinfected species of *Rickettsia* [31,49], altering tick host-seeking behavior [50,51], and affecting the progression of disease symptoms when infecting humans [52,53]. This is the first review of *R. amblyommatis*. In this review, we compiled all known information on *R. amblyommatis* in published studies, specifically discuss its distribution, discovery in different tick species, and pathogenicity, examined phylogenetic relatedness of the various strains of *R. amblyommatis* detected in ticks by PCR and from isolates of *R. amblyommatis* globally, and consider possible future directions of research needed.



Figure 1. World map displaying the distribution of *Rickettsia amblyommatis*-infected ticks. Map created using www.mapchart.net (accessed on 27 January 2023).

Table 1. Summary of quantitative data obtained from peer-reviewed publications for *R. amblyommatis*-infected wild ticks collected globally. This table includes the location in which they were collected, the species of tick, the infection prevalence, and the collection type. Studies were included if *R. amblyommatis* was confirmed through conventional PCR and sequencing analysis.

Location	Species of Tick	Infection Prevalence % [a]	Collection Type	Reference
AR, USA	*A. maculatum*	28% (58/207)	Off-host (white-tailed deer, dogs)	[54]
AR, USA	*A. americanum*	41.9% (463) [b]	Vegetation	[55]
AR, USA	*A. americanum*, *A. maculatum*, *I. scapularis*, *Amblyomma* spp.	NA [c]	Off-host (white-tailed deer, dogs)	[56]
AR, USA	*A. americanum*	98% (42/43 pools)	Flagging, CO_2 Trapping	[57]
FL, USA	*A. americanum*	29% (391/1312)	Flagging	[58]
FL, USA	*A. americanum*	29.5% (52/176)	Dragging	[50]
FL, USA	*A. americanum*	38.0% (223/588)	Dragging	[51]
FL, USA	*A. americanum*	57.1% (845/1479)	Flagging	[59]
FL, USA	*A. americanum*	37.1% (56/151)	Flagging	[47]
FL, USA	*A. americanum*, *D. variabilis*	57.3% (63/110), 35% (6/17)	Off-host (wild pig)	[60]
FL, USA	*A. americanum*, *A. maculatum*	27.8% (5/18), 16.7% (1/6)	Black bears	[61]
GA, USA	*A. americanum*	0.52% (1/194)	Dogs	[62]
GA, USA	*A. americanum*, *D. variabilis*, *A. maculatum*	27.5% (117/426), 1.4% (2/142), 9.1% (4/22)	Off-host (humans)	[53]
GA, USA	*A. americanum*	87% of pools (519/598)	Flagging	[63]
GA, USA	*A. americanum*	44.7% (315/704)	Flagging	[47]
Guam, Marianna Islands, USA	*A. breviscutatum*, *R. microplus*	2.67% (3/112)	Off-host (wild pig, Philippine deer)	[64]
IA, USA	*A. americanum*	57.9% (11/19)	Flagging	[47]
KS, USA	*A. americanum*, *D. variabilis*	93% nymphal pools, 94% adult pools, and 4.7% (169)	Dragging	[65]
KY, USA	*A. americanum*, *D. variabilis*	27.8% (30/108), 1.1% (2/179)	Off-host (canine, hog, horse, raccoon, white-tailed deer, human)	[66]
KY, USA	*D. variabilis*	0.8% (1/124)	Unknown [e]	[67]
LA, USA	*A. americanum*	60% (3/5)	Off-host (black bears)	[41]
MD, USA	*A. americanum*	87.9% (29/33 pools), 90% (27/30 individuals)	Flagging	[68]
MD, USA	*A. americanum*	64.50% [d]	Dragging	[69]
MO, USA	*A. americanum*	3.2% (40/1269)	Field surveillance	[70]
MO, USA	*A. americanum*, *D. variabilis*, *I. scapularis*	89% (24/27 pools), 100% (7/7 pools), 50% (1/2)	Flagging	[71]
MO, USA	*A. americanum*	74% (213/288)	Dragging, CO_2 bait traps, Off-host (human)	[72]
MS, USA	*A. americanum*	92.4% (73/79)	Dragging	[73]
NC, USA	*A. americanum*, *A. maculatum*, *D. variabilis*, *I. scapularis*	90.9% (412/453), 28.6% (2/7), 20% (1/5), 8.3% (1/12)	Off-host (humans)	[40]
NC, USA	*D. variabilis*	29.3% (156/532)	Flagging	[74]
NC, USA	*A. americanum*, *D. variabilis*	56.4% (871/1590), 11.1% (4/36)	Flagging	[75]
NC, USA	*A. americanum*	55.2% (216/391)	Flagging	[47]
Northeast, USA	*D. variabilis*	1.7% (3/181)	Off-host (dogs)	[76]
NJ, USA	*A. americanum*	6.6% (8/121)	Flagging	[47]
NJ, USA	*A. americanum*	25% (465/1858)	Dragging	[77]
NJ, USA	*A. americanum*	20% (49/245)	Sweeping vegetation	[78]
NJ, USA	*A. americanum*	12.8% (36/281)	Dragging and standard walking	[79]
NY, USA	*A. americanum*	58.35% (394/676)	Flagging	[80]
NY, USA	*D. variabilis*	8.3% (1/12)	Unknown [e]	[67]
NY, USA	*A. americanum*	41.7% (198/475)	Flagging	[47]
OH, USA	*A. americanum*	30.2% (93/308)	Off-host (humans), Flagging	[81]

Table 1. *Cont.*

Location	Species of Tick	Infection Prevalence % [a]	Collection Type	Reference
OH, USA	*A. americanum*	38% (8/21)	Humans	[82]
OK, USA	*A. americanum*	10% (6/60)	Flagging	[47]
OK, USA	*A. americanum*	33.6% (146/434)	Unknown [e]	[83]
OK, USA	*A. americanum*	17.6% (123/700)	Dry Ice Traps	[84]
RI, USA	*A. americanum*	47.4% (18/38)	Flagging	[47]
SC, USA	*A. americanum*	11.7% (545) [b]	Off-Vegetation	[55]
SC, USA	*A. americanum*	45.6% (36/79)	Flagging	[47]
SC, USA	*A. americanum*, *D. variabilis*	80% (84/105), 66.7% (10/15)	Off-host (Wild Pig)	[60]
TN, USA	*A. americanum*	16.6% (96) [b]	Vegetation	[55]
TN, USA	*A. americanum*, *D. variabilis*	40% (255/655), 2.5% (14/555)	Off-host (humans, wild animals, dogs)	[85]
TN, USA	*A. americanum*	4.9% (45/926)	Dragging, CO_2 baited trap	[86]
TX, USA	*A. auricularium*, *A. geayi*, *A. longirostre*	28.6% (2/7), 33.3% (1/3), 11.9% (5/42)	Off-host (wild birds)	[87]
TX, USA	*A. americanum*, *I. scapularis*	50% (41/82), 1.47% (2/136)	Dragging	[88]
TX, USA	*A. americanum*	100% (68/68 pools)	Dry ice trapping, Dragging, and Flagging	[89]
TX, USA	*A. inornatum*	100% (3/3)	Off-host (deer)	[90]
TX, USA	*A. inornatum*	Unknown [g]	CO_2 Traps	[91]
TX, USA	*A. americanum*, *A. cajennense*	30.3% (179), 32.3% (10) [f]	Off-host (humans)	[92]
VA, USA	*A. americanum*	72.8% (pools)	Dragging	[93]
VA, USA	*A. americanum*	80.3% of pools [d]	Flagging	[94]
VA, USA	*D. variabilis*	4.2% (9/214)	Unknown [e]	[67]
VA, USA	*A. americanum*	19% (65/340)	Dragging	[95]
USA	*D. variabilis*	0.1% (7/5286)	Off-host (humans)	[96]
USA	*A. americanum*	80.5% (58/72 pools), 66.5% (244/367)	Off-host (humans)	[48]
Córdoba, Argentina	*A. neumanni*	23.6 (13/55)	Walking Survey, CO_2 traps	[97]
Northern Argentina	*A. neumanni*	21% (3/14)	Off-host and Flagging	[98]
Salta, Argentina	*A. hadanii*, *A. neumannii*	21.3% (27/127), 44.4% (8/18)	Dragging	[99]
Salta, Argentina	*A. tonelliae*	1.47% (1/68)	Dragging	[100]
Argentina	*A. hadanii*	1.6% (1/60)	Vegetation and Off-host (cattle)	[101]
Australia	*I. tasmani*	28.2% (22/78)	Off-host (koalas)	[102]
Belize	*A. pacae*	100% (1/1)	Off-host (spotted paca)	[103]
Belize	*A. cajennense*, *A. maculatum*	55.1% (81/147), 66.7% (2/3)	Off-host (dog)	[104]
Acre, Brazil	*A. humerale*	33.3% (1/3) of A. humerale	Off-host (capybara)	[105]
Acre, Brazil	*A. longirostre*, *A. geayi*	62.5% (5/8), 50% (1/2)	Off-host (wild birds)	[106]
Acre, Brazil	*A. rotundatum*	14.3% (1/7)	Off-host (Amazon tree boa)	[107]
Amazonas, Brazil	*A. humerale*	2.17% (1/46)	Off-host (tegu)	[108]
Bahia, Brazil	*A. longirostre*	15.4% (2/13)	Off-host (wild birds)	[109]
Bahia, Brazil	*A. longirostre*, *A. varium*	24.4% (11/45), 20% (1/5)	Off-host (wild birds)	[110]
Bahia, Brazil	*A. longirostre*	23.7% (9/38)	Off-host (thin-spined porcupine and hairy dwarf porcupine)	[111]
Bahia, Brazil	*A. auricularium*, *Ambylomma* spp. larvae	57.5% (46/80)	Off-host (gray short-tailed opossum, rodent, Brazilian three-banded armadillo)	[112]
Espírito Santo, Brazil	*A. humerale*	50% (5/10)	Off-host (tortoise)	[113]
Maranhão, Brazil	*A. cajennense*	1% (1/100)	Off-host (dogs)	[114]
Maranhão, Brazil	*A. cajennense*	16.7% (3/18)	Off-host (horses)	[115]
Mato Grosso, Brazil	*A. cajennense*	0.45% (3/665)	Off-host (horse, donkey)	[116]
Mato Grosso, Brazil	*A. cajennense*	31% (5/16 pools)	Off-host (human) and flagging	[117]

Table 1. *Cont.*

Location	Species of Tick	Infection Prevalence % [a]	Collection Type	Reference
Mato Grosso, Brazil	*A. auricularium*, *A. cajennense*	100% (1/1), 69.4% (136/196)	Off-host (wild animals)	[118]
Mato Grosso, Brazil	*A. cajennense*, *A. ovale*	3.3% (5/152), 15.4% (2/13)	Off-host (dogs)	[119]
Mato Grosso, Brazil	*A. cajennense*, *A. coelebs*	38.4% (10/26), 70% (7/10)	Off-host	[120]
Mato Grosso, Brazil	*A. sculptum*	1.2% (1/85 pools)	Off-host (Azara's agouti)	[121]
Mato Grosso, Brazil	*A. longirostre*	45.5% (5/9)	Off-host (wild birds)	[122]
Mato Grosso, Brazil	*A. dubitatum*	5% (1/20)	Off-host (dog)	[123]
Mato Grosso, Brazil	*A. cajennense*, *A. coelebs*, *A. humerale*, *Amblyomma* spp.	NA [g]	Off-host (rodents, marsupials)	[124]
Minas Gerais, Brazil	*A. longirostre*	(13/49; 26%)	Off-host (wild birds)	[125]
Pará, Brazil	*A. longirostre*, *A. humerale.*	70% (7/10), 22.2 % (2/9)	Off-host (wild animals)	[118]
Pará, Brazil	*A. longirostre*, *A. geayi*	56.7% (38/67), 57.1% (4/7)	Off-host (birds)	[126]
Paraíba, Brazil	*A. auricularium*, *A. longirostre*	23.1% (3/13), 45.5% (10/22)	Off-host (wild birds)	[127]
Paraná, Brazil	*A. longirostre*	32.3% (11/34)	Off-host (wild Birds)	[128]
Paraná, Brazil	*A. coelebs*	1.9% (8/420)	Off-host (ring-tailed coatis)	[129]
Pernambuco, Brazil	*A. auricularium*	20% (1/5)	Off-host (rodent)	[130]
Pernambuco, Brazil	*A. auricularium*	53.3% (8/15)	Off-host (skunk, rabbit)	[131]
Pernambuco, Brazil	*A. pseudoconcolor*	90.9% (10/11)	Off-host (dog, six-banded armadillo)	[132]
Rio Grande do Norte, Brazil	*A. auricularium*	11.1% (14/126 individuals and pools)	Off-host (white-eared opossum and xix-banded armadillo)	[133]
Rio Grande do Sul, Brazil	*A. longirostre*	66.7% (2/3)	Off-vegetation, Dragging	[134]
Rondônia, Brazil	*A. coelebs*, *A. cajennense*	14.3% (1/7), 26.8% (11/41)	Off-vegetation	[135]
Rondônia, Brazil	*A. cajennense*, *A. oblongoguttatum*	6.3% (2/32) + 2 pools, 16.7% (1/6)	CO_2 traps; drag flagging; off-vegetation	[136]
Santa Catarina, Brazil	*A. longirostre*	100% (1/1)	Unknown [e]	[137]
São Paulo, Brazil	*A. coelebs*	Unknown [g]	Off-vegetation	[138]
Brazil	*A. calcaratum*, *A. longirostre*	50% (1/2), 12.3% (9/73)	Off-host (wild Birds)	[139]
Brazil	*A. sculptum*	0.5% (1/200)	Dragging	[140]
Chile	*Ornithodoros* sp.	100% (4/4)	Off-host (rodent)	[141]
Caldas, Colombia	*A. longirostre*, *A. varium*, *Ixodes* sp.	6.67% (3/45), 66.6% (2/3), 11.1% (1/9)	Off-host (wild Birds)	[142]
Villeta, Colombia	*A. cajennense*	9.1% (1/11)	Off-host (humans)	[143]
Costa Rica	*A. cajennense*	66.7% (10/15)	Off-host (horse)	[144]
Costa Rica	*A. mixtum*, *A. ovale*, *D. nitens*, *R. sanguineus*	NA [g]	Off-host (horses, cows, humans, dogs) and off-vegetation	[145]
Costa Rica	*R. sanguineus*	16.7% (1/6 pools)	Off-host (dogs)	[146]
Costa Rica	*A. longirostre*, *A. geayi*	7.4% (4/54 pools), 3.7% (2/54 pools)	Off-host (wild birds)	[147]
Cuba	*A. mixtum*	35.7% (5/14)	Off-host (horses)	[148]
Cuba	*A. mixtum*, *Amblyomma* spp.	73% pools (422) [f]	Off-host (horses and dogs)	[149]
El Salvador	*A. mixtum*, *A. parvum*, *D. nitens*, *Amblyomma* spp.	77% (10/13), 50% (8/16), 8% (1/13), 11% (1/9)	Off-host (wild and domestic animals)	[150]
Regina, French Guiana	*A. coelebs*	15.4% (2/13)	Off-host (white-lipped peccaries)	[151]
French Guiana	*A. cajennense*, many more	25% [f], NA [g,f]	Dragging, Flagging, Off-host	[152]

Table 1. *Cont.*

Location	Species of Tick	Infection Prevalence % [a]	Collection Type	Reference
Honduras	*A. longirostre*, *A. mixtum*	14% (1/7), 80% (4/5)	Off-host (wild birds, humans)	[153]
Reynosa, Mexico	*R. sanguineus*	1% (3/292)	Off-host (domestic dogs)	[154]
Mexico	*A. mixtum*, *A. oblongoguttatum*, *A. parvum*	33.5% (59/176), 3.17% (2/63), 5.7% (3/53)	Off-vegetation, Off-host (possum, ocelot, white-nosed coati)	[155]
Mexico	*A. maculatum*, *A. mixtum*	9.8% (5/51), 13.2% (17/129)	Off-host (wild pigs)	[156]
Mexico	*A. mixtum*	50% (1/2)	Off-host (iguana)	[157]
Mexico	*A. mixtum*	40% (2/5)	Human	[158]
Nicaragua	*A. ovale*	100% (1/1 pools)	Off-host (dogs)	[146]
Pakistan	*R. microplus*, *H. anatolicum*, *and H. dromedarii*	4%(21/514) [f]	Off-host (buffalo, cattle, sheep, goat, donkey, camel)	[159]
Panama	*A. mixtum*, *H. juxtakochi*, *Amblyomma* spp.	27.8% (5/18), 4.3% (1/23), 8.2% (12/146 pools)	Dragging	[160]
Panama	*A. mixtum*	94.1% (48/51)	Off-host (tapir, horses)	[42]
Panama	*A. cajennense*, *A. oblongoguttatum*, *A. ovale*, *R. sanguineus*	86.4% (38/44), 50% (2/4), 27.9% (12/43), 3.1% (2/64)	Off-host (dogs, horses, pig)	[161]
Panama	*A. cajennense*, *D. nitens*, *R. sanguineus*	36.7% (11/30), 27.4% (17/62), 12.3% (7/57)	Off-host (horses, dogs)	[162]
Panama	*A. mixtum*, *H. Juxtakochi*, Immature *Amblyomma* spp.	27.8% (5/18), 4.6% (3/65), 11.5% (12/104)	Dragging	[160]
Paraguay	*A. longirostre*	11.8% (2/17)	Off-host (wild birds)	[163]

[a] An infection prevalence % is calculated by dividing the number of ticks (in each species) reported to test positive for *R. amblyommatis* by the total number of ticks collected of that species and multiplying by 100. Values are listed in the respective order of the species listed in the species column. [b] Unable to locate the number of ticks infected in publication. [c] Unable to report infection prevalence % as not all positive tick samples were sequenced in this study. [d] This value is an average infection prevalence over a two-year period. [e] The publication did not specify the collection type. [f] The total number of ticks collected for each species not reported in the study. [g] Unable to calculate infection prevalence % with the information given in the study.

4. Distribution and Spread of *Rickettsia amblyommatis*

4.1. Tick Vectors

Rickettsia amblyommatis was reported in 34 species of ticks globally, with the majority within the genus *Amblyomma*. Figure 2 illustrates the proportion of each species of tick infected with *R. amblyommatis* reported in the literature. *Amblyomma* spp. made up about 83% of the ticks reported to be infected with *R. amblyommatis* (Figure 2). Through this literature search, *A. americanum* was found to be the most common species of tick infected with *R. amblyommatis*, making up about 27.8% of the reported tick species; *A. americanum* populations infection rates ranged from 1 to 90% (Table 1) [40,47,48,50,51,58–60,63,65,66,69–71,75,81–83,85,92,93,164]. Studies of the microbiome of ticks have found *R. amblyommatis* to be present in *A. americanum* and *A. maculatum*, specifically finding *R. amblyommatis* to be the most common member of Rickettsiales present within the lone star tick [164,165].

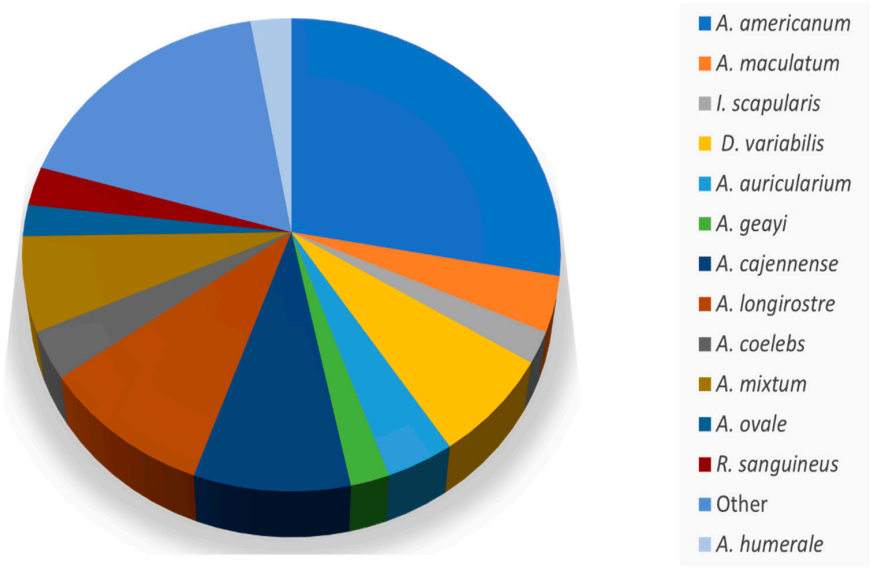


Figure 2. A pie graph of the species of tick reported infected with *Rickettsia amblyommatis*. The legend labeled as "other" included species that were only seen reported in 1–3 instances. These species were as follows: *A. breviscutatum*, *A. calcaratum*, *A. dubitatum*, *A. hadanii*, *A. humerale*, *A. inornatum*, *A. neumanni*, *A. oblongoguttatum*, *A. pacae*, *A. parvum*, *A. pseudoconcolor*, *A. rotundatum*, *A. tonelliae*, *A. varium*, *D. nitens*, *H. anatolicum*, *H. juxtakochi*, *Hyalomma dromedarii*, *Ornithodoros* sp., *I. tasmani*, and *R. microplus*.

Amblyomma americanum is a human-biting tick and competent vector of the causative agents of RMSF, Human Monocytic Ehrlichiosis, tularemia, and heartland virus, and it is more recently known to cause a meat allergy in humans [45,166,167]. While *A. americanum* is native to the Southeastern United States, this tick is found to be significantly expanding in geographic range, perhaps due to a combination of its generalist tendencies and changing climatic conditions, which will likely further escalate the rise in tick-borne diseases [13,46,168–171]. This northward expansion has been so successful that *A. americanum* is now the most abundant species of tick in Long Island, New York [46,172–174]. This expansion and growth in populations could lead to shifts in disease prevalence by the possible displacement of other species of tick [80]. Due to *A. americanum*'s aggressive nature, successful expansion, and high rate of *R. amblyommatis* infection, it has been predicted that the number of infections of *R. amblyommatis* in people would increase with time [1,9,45–47,168]. However, in clinical settings, there is a lack of *Rickettsia* species-specific serological testing making it unclear if this prediction is occurring [34]. *Amblyomma longirostre* was the 2nd most common species of tick found to be infected with *R. amblyommatis*, making up about 10% of the infected species of tick reported. This species of tick has a geographic range from southern Mexico to Argentina with a wide distribution throughout Brazil [175].

Past studies have shown that ticks that are infected with a pathogen can experience changes in behavior, physiology, and survivability [176–179]. When a tick is infected with *R. amblyommatis*, the bacteria can be found in the ovaries, midgut, and salivary glands [9,180–183]. Ticks infected with *R. amblyommatis* displayed changes in their host-seeking behavior, such as spending less time questing compared to uninfected ticks [50,51]. It is unclear how this will affect the transmission dynamics and spread of *R. amblyommatis*. It is possible that infection with *R. amblyommatis* could cause a disruption in water balance dynamics in *A. americanum*, making ticks more susceptible to desiccation and preventing them from spending as much time host-seeking [51,177,184,185].

4.2. Geographic Range

In the USA, *R. amblyommatis* has a wide geographic range, being identified in ticks as far south as Florida, as far west as Kansas, and as far north as New York [47,65] (Figure 1; Table 1). Outside of the United States, *R. amblyommatis* was detected in Brazil, Argentina, Australia, Belize, Chile,

Panama, Colombia, Costa Rica, Cuba, Mexico, Nicaragua, Honduras, Paraguay, Pakistan, French Guiana, and El Salvador [97,102,103,110,111,118,126,141,142,147,149,150,153,156,159,160,163,186] (Figure 1; Table 1). In Table 1, the geographic range of *R. amblyommatis* for different tick species and the percentage infected in the population sampled are summarized. Additionally, Bermudez and Troyo (2018) concluded that this species was the most common rickettsial species in Central America [187].

In North Carolina, USA, it was concluded that *R. amblyommatis* is the most common rickettsial exposure that humans experience after being bitten by a lone star tick [40]. These data give validity to the suggestion that "*R. amblyommatis* likely represents the most prevalent and widely distributed SFG rickettsial species in the Americas" [38]. There are several plausible explanations for the widespread distribution of *R. amblyommatis*; however, one possible factor explaining the geographic expansion of *R. amblyommatis* is likely the resurgence and expansion of white-tailed deer populations; this mammal is the preferred host of *A. americanum* [45,48,188,189]. Since *A. americanum* is the most frequent carrier of *R. amblyommatis*, movement by deer may have led to the geographic expansion of *R. amblyommatis*. Ticks are considered both the reservoir and the vector for *R. amblyommatis* which causes quicker and further spread of this spotted fever species as it does not have to depend on hosts to proliferate [27,55,131,189,190] and, indeed, the geography of tick-borne rickettsioses such as *R. amblyommatis*, may be mainly based on the distribution of the vector tick species [131,190]. Another aspect that could be helping fuel this spread could be *Amblyomma* spp.'s generalist tendencies when finding a host, increasing their likelihood of survival by finding a host quickly and moving to the next life stage [191].

4.3. Hosts of Rickettsia amblyommatis-Infected Ticks

Workers in the majority of field studies that have detected *R. amblyommatis* in tick populations have collected unfed host-seeking ticks while dragging or flagging. However, in some cases, ticks were collected from wild or domestic animals. These data provide some information about possible vertebrate reservoir hosts of *R. amblyommatis* and could help determine the geographic distribution of this *Rickettsia* species. While some tick-borne bacterial pathogens have known reservoirs, *R. amblyommatis* does not; as stated earlier, some have hypothesized that there is no vertebrate reservoir [55,189]. For example, Apperson et al. (2008) reported white-tailed deer to be negative for antibodies to *R. amplyommii*, suggesting deer are not becoming infected with this rickettsial species by lone star ticks [189]. We have compiled a list of all of the animals found with feeding ticks infected with *R. amblyommatis* (Table 1). This list includes a wide variety of animals, with the most common including humans, wild birds, dogs, horses, deer, and others (Table 1). Birds in particular play an important role in tick-borne diseases by spreading pathogenic bacteria over long distances to new areas [122,147,192,193]. Studies on infected ticks feeding on birds have the potential to explain some of the distribution patterns of *R. amblyommatis*. In a study carried out by Dolz et al. [147], most *R. amblyommatis*-infected ticks were found on resident passerine birds that did not migrate. However, one *R. amblyommatis*-infected tick was found on *Catharus ustulatus*, a thrush that migrates far distances such as from the USA to Argentina. This finding reveals that migrating birds could potentially play a role in the dispersion of *R. amblyommatis* over long distances. Additionally, Budachetri et al. [194] investigated the microbiome of birds and the ticks infesting them and did not find similarities between the microbiomes, leading them to conclude that migratory birds do not act as a reservoir host for *R. amblyommatis* but rather could possibly play a role as a means of dispersal for ticks and tick-borne pathogens. Mukherjee et al. [193], who conducted a study on exotic ticks infesting migrating songbirds, found these ticks to harbor high levels of *Rickettsia* spp. including *R. amblyommatis* and concluded that it is likely that birds can transport these ticks and tick-borne pathogens long distances.

5. Pathogenicity

There has long been conflicting information on whether *R. amblyommatis* is pathogenic to mammals. In 1981, Burgdorfer was unable to create rickettsial disease in guinea pigs when experimentally infected with *R. amblyommatis*; however, he found that this infection caused mild symptoms in meadow voles (*Microtus pennsylvanicus*) [55]. Despite that, the author concluded that *R. amblyommatis* was not pathogenic to humans due to the lack of symptoms such as a rash, headache, or fever reported by people in areas where *R. amblyommatis* tick infection prevalence was high. Since then, more studies have been conducted that have indicated that *R. amblyommatis* could induce illness in mammals. For example, mice that were intravenously infected with *R. amblyommatis* were found to have manifested mild disease, showing clinical symptoms such as weight loss [9]. However, it was seen that *R. amblyommatis* displayed an attachment deficiency to microvascular endothelial cells resulting in a slow growth rate, suggesting that mice would require high titers of *R. amblyommatis* to become infected and manifest symptoms of disease [9]. Similarly, Snellgrove et al. [195] found that when guinea pigs were inoculated with *R. amblyommatis*, some of the guinea pigs developed fever, orchitis, and dermatitis. Rivas et al. [196] found that when guinea pigs were inoculated with *R. amblyommatis*, the guinea pigs experienced an immune response in the form of antibody development, and two out of six of the animals displayed testicular alterations; however, no fever, weight loss, or other signs of infection were exhibited. Levin et al. [180] reported that guinea pigs developed a disseminated infection when fed upon by ticks infected with *R. amblyommatis*. *Rickettsia rickettsii* has also been shown to have highly varied levels of virulence in animal models; therefore, it is not surprising that this is the case with *R. amblyommatis* [9,197,198]. It is also possible that these differences in observed pathogenicity in guinea pigs could be due to varied strains of *R. amblyommatis* being used in the experiments [9].

While most researchers did not conduct serological testing on animals with attached ticks, there were some exceptions as well as studies on animals artificially infected with *R. amblyommatis* to evaluate transmission potential. Some studies did not report any clinical symptoms but were able to demonstrate the transmission of *R. amblyommatis* from infected ticks to the animals by detecting seroactivity, such as antibody titers to *R. amblyommatis* in rural dogs (*Canis familiaris*), rabbits (*Oryctolagus cuniculus*), white-eared possums (*Didelphis albiventris*), the black rat (*Rattus rattus*), and *R. amblyommatis* DNA was found in a single case in the blood of a dog [84,114,131,133,134,146,186,199]. The majority of the results from these mammal studies show a consistent pattern that supports the premise that *R. amblyommatis* may cause a self-limiting mild febrile illness in mammals and humans, with immune-compromised individuals being particularly vulnerable to infection with this pathogen [9,196].

5.1. Rickettsia amblyommatis in Humans

Little information is available on cases of *R. amblyommatis* infection in humans. While there are increasing numbers of cases of RMSF-like illnesses, there is a lack of detection of *R. rickettsii* in tick surveillance studies, suggesting that these illnesses are associated with the expanding range of *A. americanum* and possibly the transmission of *R. amblyommatis* [46,80]. *Rickettsia amblyommatis* being a prevalent bacterium does not necessarily prove that this species is causing disease in humans; however, studies mentioned previously have implicated that infection with *R. amblyommatis* is correlated with symptoms of disease. For example, one human patient was found to have a rash at a tick bite site, and PCR testing on the tick found it to be infected with *R. amblyommatis*, making this the first known association of *R. amblyommatis* with a rash [52]. The sequencing data obtained had an identical match to *R. amblyommatis*, accession number EF063690 [52]. Billeter et al. [52] discussed a situation where soldiers became ill with febrile symptoms after working in *A. americanum*-infested sites and stated that serological evidence indicated that infection with *R. amblyommatis* was the possible cause. In Georgia, USA, twelve residents experiencing tick bites met the criteria for a possible tick-borne illness by having ticks attached for 6 h or more and

manifested clinical symptoms of a tick-borne illness after a 4-d incubation [53]. These residents obtained PCR testing of their attached ticks and eight were found to be infected with *R. amblyommatis*. Two of these individuals who had an *R. amblyommatis*-infected tick bite reported a bulls-eye rash (erythema migrans) [53]. However, these individuals had multiple ticks attached to them, and it could not be determined which tick was originally at the site of the rash. In North Carolina, USA, outdoor workers are experiencing an increased risk of tick-borne infections; in one serologic study, seroconversions to *R. amblyommatis* were demonstrated, indicating infection, but no symptoms were recorded in the workers [200]. While there have not been any cases where *R. amblyommatis* was isolated from human patients, serological evidence indicates that this species of *Rickettsia* could be the cause of human illnesses displayed as RMSF-like symptoms [9].

Apperson et al. [189] collected ticks in North Carolina, USA, where human cases of RMSF were high. The collected ticks were molecularly tested for bacterial pathogens and 11 out of 25 *A. americanum* pools were found to be positive for *R. amblyommatis* [189]. Human patients from the same county that were presumptively diagnosed with a tick-borne illness were serologically tested, and three out of six patients were diagnosed as probable RMSF cases showing a greater increase in IgG-class antibody titers between paired acute and convalescent sera to *R. amblyommatis* antigens but not to *R. rickettsii* antigens [189]. These results suggest that human cases of spotted fever rickettsiosis diagnosed as RMSF may actually be experiencing an infection with *R. amblyommatis*. The authors felt that further investigations were needed into the pathogenicity of *R. amblyommatis* in humans. Other studies have also suggested that *R. amblyommatis* is a human pathogen causing misdiagnoses with other rickettsial spotted fever illnesses such as RMSF [47,201,202].

The studies that have been discussed show that it is possible that *R. amblyommatis* is pathogenic and can result in clinical symptoms in humans and animals. However, an infection with this pathogen may result in a minor, self-limiting illness in most individuals, especially those who are immune compromised [9,196]. It is also worth noting that when attempting to diagnose rickettsial infections, there may be confounding effects as all SFG rickettsia induce cross-reading antibodies [35]. With that in mind, previous infection with *R. amblyommatis* could be mistakenly diagnosed as RMSF during serological testing. Another hypothesis is that simultaneous infection with *R. amblyommatis* and other spotted fever group rickettsiae species would result in a lack of clinical symptoms due to this coinfection providing cross-immunity [145,147]. Other species of *Rickettsia* have overlapping geographic ranges with *R. amblyommatis*, such as *R. parkeri*, which is considered pathogenic to humans [203]. This could result in issues during diagnosis. While there is serological confirmation of ticks infected with *R. amblyommatis* that have bitten humans, there is still no direct evidence that *R. amblyommatis* may cause human infection.

5.2. Coinfection Effects and Inhibition

Over 25 years ago, Telford et al. [204] warned of the unique impact coinfections could have on public health with a growing number of highly widespread diverse tick-borne pathogens. With *A. americanum* experiencing a markedly expanding geographic range, this could affect the likelihood of coinfections. Recent studies have shown that when *R. rickettsii* (RMSF) is coinfected with *R. amblyommatis*, the latter may act as a sort of protection, lowering the severity of RMSF through an elicited immune response. Blanton et al. [31] found that guinea pigs who were initially inoculated with *R. amblyommatis* did not become ill when exposed to *R. rickettsii* but guinea pigs without previous exposure to *R. amblyommatis* did become ill from *R. rickettsii*. This study provided evidence for the potential protective role that *R. ambyommatis* plays in the epidemiology of RMSF. A similar finding was reported by Wright et al. [49], where *A. americanum* was infected with *R. amblyommii* and was less likely to develop an *R. parkeri* infection when feeding alongside an *R. parkeri*-infected *A. maculatum*. Additionally, it was seen that *R. amblyommatis*-infected *A. americanum* were less likely to become infected nor maintain an infection of *R. parkeri* [49]. These studies show that it is likely that *R. amblyommatis* is displaying a form of rickettsial exclusion, making

coinfection with another rickettsial species less likely [55,139]. These findings suggest that an increase in infection prevalence of *R. amblyommatis* in ticks might decrease cases of RMSF. Interestingly, after two decades of increasing cases of RMSF, in 2018 and 2019 there was a slight decrease in reported cases of RMSF [34]. Similar protective effects when *R. amblyommatis* is coinfected with other *Rickettsia* species might also occur; *A. americanum*, the main transmitter of *R. amblyommatis*, also transmits other pathogens [205]. Investigations into the effects of *R. amblyommatis* coinfection with other tick-borne pathogens affecting virulence, transmissibility, and clinical symptoms in hosts are clearly warranted.

6. Phylogenetic Analysis

The cultivation of *Rickettsia* species can vary depending on the cell line and culture system used. Sayler et al. [59] found that when culturing tick-borne organisms, it was best to use the cell lines acquired from the tick vector itself. *Rickettsia amblyommatis* has been propagated in the past in tick cell lines, mosquito cell lines, Vero cells, tissue cells, chicken fibroblast, primary embryonated chicken eggs, and the HUVEC line [38,59,69,135,206]. Karpathy et al. [38] established a pure culture of strain WB-8-2T (=CRIRC RAM004T=CSURP2882T) and assigned it as the type strain for *R. amblyommatis*. Prior to this, the species was isolated from *A. americanum* by Stothard et al. [44]. It was seen that this isolate was similar to other species of *Rickettsia* but proved to be unique through the analysis of the 17 kDa antigen gene [38]. The genome of *R. amblyommatis* of the strain GAT-3OV has been sequenced and submitted under the accession number NC_017028. The phylogenetic analysis completed by Karpathy et al. [38] was concatenated using five gene regions (*rrs*, *gltA*, *sca0*, *sca5*, and *sca4*) and showed that the most closely related *Rickettsia* species to *R. amblyommatis* were *R. japonica* and *R. raoultii*.

Past studies have hypothesized that various strains of *R. amblyommatis* could be genetically independent, with differences evolving based on the tick species and geographic location [99,190]. Dolz et al. [147] found that Costa Rican strains of *R. amblyommatis* showed intraspecific variation and found high associations with strains from some South American countries. With that in mind, we sought to create a phylogenetic tree of the *R. amblyommatis* sequences that were present in the NCBI database to compare strains and investigate the relationship between strains, location, and tick species. We created two trees, one that focused on the gene targeting the outer membrane protein A (*ompA*) and a second targeting outer membrane protein B (*ompB*). Sequences focusing on these two gene regions were selected as they have been noted to be polymorphic, with *ompB* being suitable for analyzing intraspecific differences in *R. amblyommatis* [190]. The methods involved in the creation of the phylogenetic trees can be seen in the Materials and Methods section.

In total, there were 104 sequences identified for *ompA*; however, 25 (24.04%) were not included due to their small size; only sequences greater than 406 base pairs were included in the analysis. The pairwise comparison of *ompA* sequences revealed similarity values between 97.27% and 100% among strains. For the analysis of *ompB*, 19 sequences were incorporated, and only sequences greater than 600 base pairs were included for a total of 618 base pairs in the final tree. The pairwise comparison of *ompB* sequences revealed similarity values between 98.2% and 100% among strains.

Phylogenetic analysis based on *ompA* gene sequences with maximum likelihood revealed four major branches (groups) among the *R. amblyommatis* strains (Figure 3). Group 1 consisted of 56 sequences, group 2 comprised ten strains of *R. amblyommatis*, group 3 contained ten sequences of *R. amblyommatis*, and group 4 had two sequences. The *ompA* phylogenetic analysis showed that *R. amblyommatis* did not display cluster organization for clusters 1,2, and 4 but did for cluster 3. Cluster 3 appears to be organized by tick species and geographic origin of the sequences where most of the sequences came from Brazil, with two being from Costa Rica and Colombia, and the majority of the sequences were obtained from *A. longirostre*. The lack of cluster organization in the other clusters could be due to the sequence length in this analysis (Figure 3). For example, in group 1 (N = 56), *R. amblyommatis* (Aa) sequences were identified to include as many as 14 different tick species

from over eight different countries. Phylogenetic analysis of the *ompB* gene formed two groups (Figure 4). Group 1 contains 17 sequences and group 2 contains two sequences. These clusters do not appear to be organized by location or species of tick.

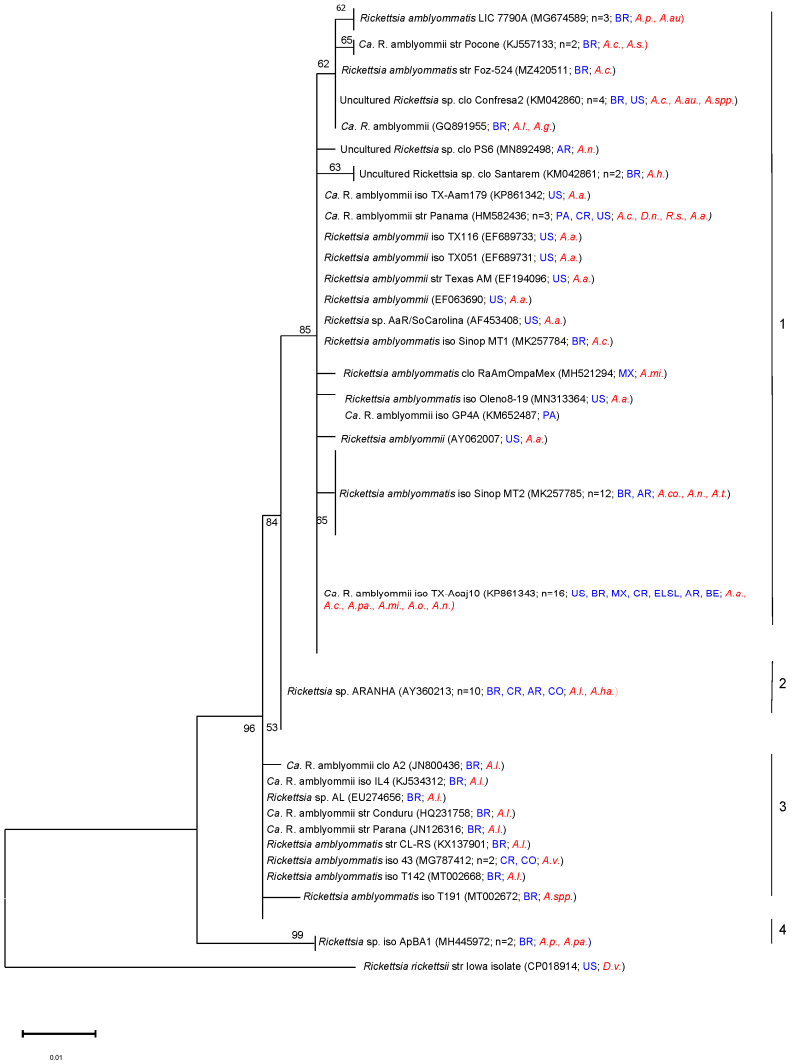


Figure 3. Phylogenetic tree of published sequences and reports of *Rickettsia amblyommatis* in ticks. The analyses were based on the sequences of the *ompA* gene region of *Rickettsia amblyommatis* within 406 bp obtained from NCBI BLAST. This tree shows a collapsed version incorporating a total of 79 nucleotide sequences (n shows the total number of sequences included in that group). The phylogenetic analysis was completed using a maximum likelihood method on the Kimura 2-parameter model (ML; bootstrap replicates: 1000). This tree is collapsed with nodes organized by sequence differences of 0.001. Instances in which publications reported the species as "*Rickettsia* spp." but indicated in the publication that the species was *R. amblyommatis* were included in this analysis. The GenBank accession numbers are given in parentheses. Scale bars indicate the number of substitutions per nucleotide position. The *Rickettsia rickettsii* Iowa isolate (CP018914) was included as an outgroup. Tick species included in this tree are as follows: *A auricularium (A.au.)*, *A. cajennense (A.c.)*, *A. coelebs (A.co.)*, *A. geayi (A.g.)*, *A. hadanii (A.ha.)*, *A. humerale (A.h.)*, *A. longirostre (A.l.)*, *A. americanum (A.a.)*, *A. mixtum (A.mi.)*, *A. neumanni (A.n.)*, *A. oblongoguttatum (A.o.)*, *A. pacae (A.pa.)*, *A. parvum (A.par.)*, *A. pseudoconcolor (A.p.)*, *A. sculptum (A.s.)*, *Amblyomma* spp. *(A.spp.)*, *A. varium (A.v.)*, *D. nitens (D.n.)*, *D. variabilis (D.v.)*, and *R. sanguineus (R.s.)*. The countries included in this tree are as follows: Brazil (BR), United States of America (US), Mexico (MX), Panama (PA), Argentina (AR), Costa Rica (CR), Belize (BE), El Salvador (ELSL), and Colombia (CO).

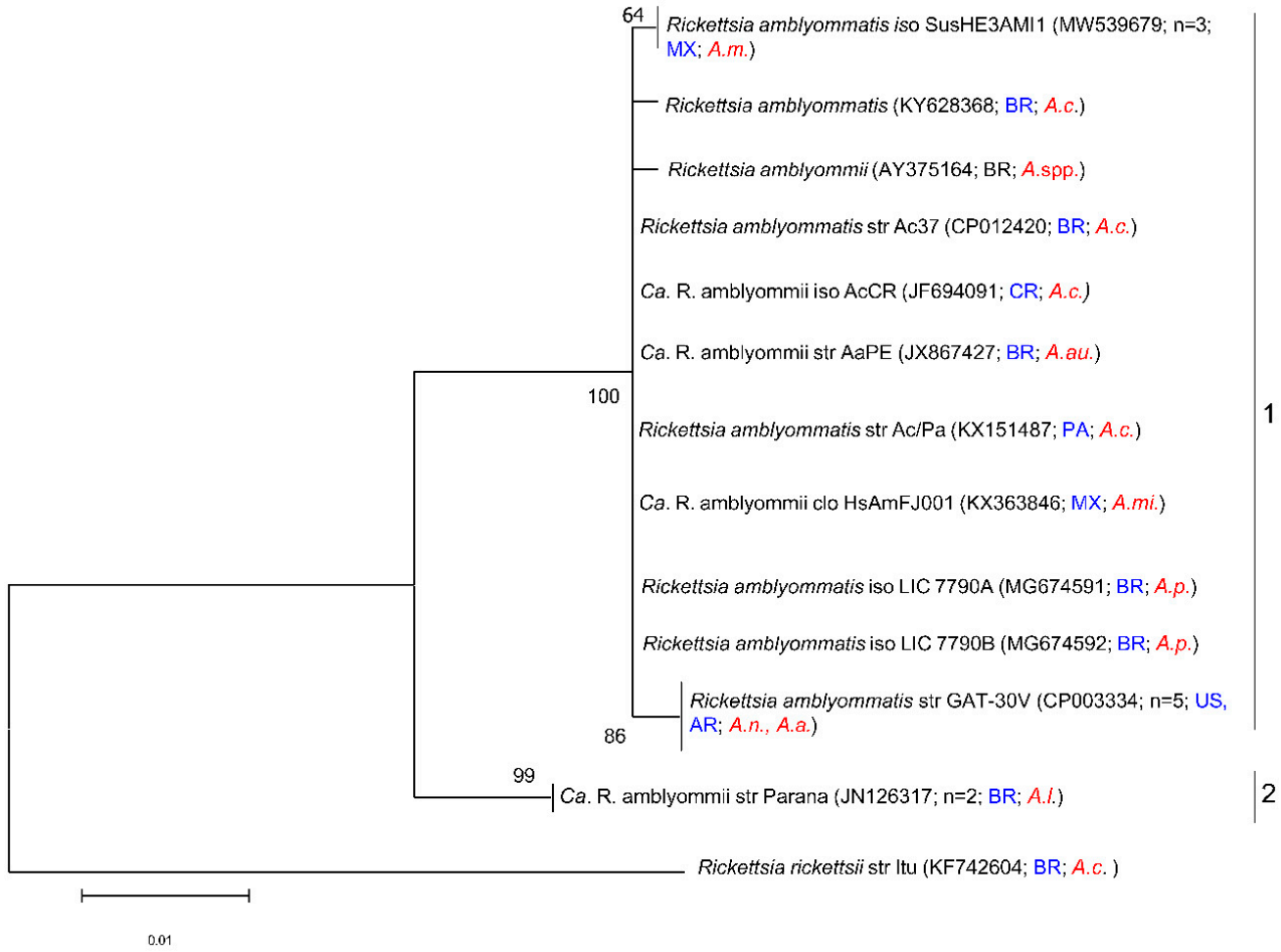


Figure 4. Phylogenetic tree of published sequences and reports of *Rickettsia amblyommatis* in ticks. The analyses were based on 20 partial sequences of the *ompB* gene region of *Rickettsia amblyommatis* within 618 bp obtained from NCBI BLAST. The phylogenetic analysis was completed using a maximum likelihood method on the Kimura 2-parameter model (ML; bootstrap replicates: 1000). This tree is collapsed with nodes organized by sequence differences of 0.001 (n shows the total number of sequences included in that group). Instances in which papers reported the species as "*Rickettsia* spp." but indicated in the publication that the species was *R. amblyommatis* were included in this analysis. The GenBank accession numbers are given in parentheses. Scale bars indicate the number of substitutions per nucleotide position. This phylogenetic tree was created using MEGA11. Tick species included in this tree are as follows: *A. americanum (A.a.)*, *A auricularium (A.au.)*, *A. cajennense (A.c.)*, *A. longirostre (A.l.)*, *A. maculatum (A.m.)*, *A. mixtum (A.mi.)*, *A. neumanni (A.n.)*, *A. pseudoconcolor (A.p.)*, and *Amblyomma* spp. *(A.spp.).* The countries included in this tree are as follows: Brazil (BR), the United States of America (US), Panama (PA), Argentina (AR), Mexico (MX), and Costa Rica (CR).

7. Conclusions and Future Directions

While there is still much that is unknown about *R. amblyommatis*, through the compilation of published work on this topic, we can see that some peer-reviewed studies have found *R. amblyommatis* to be mildly pathogenic to mammals and humans, that it influences the virulence of other pathogens when coinfected, and that there is intraspecific variation between strains of this pathogen. However, overall, there are inconsistencies, with some studies not finding proof of pathogenicity and others finding symptoms of disease associated with *R. amblyommatis*. Disease symptoms from infection have been seen in humans and laboratory animals; however, some laboratory challenge studies could not produce similar findings. *Rickettsia amblyommatis*'s status as a pathogen needs to be verified through further testing.

There remain many questions about *R. amblyommatis*, which could lead to productive research. One of the most important gaps in the knowledge is how coinfections with *R. amblyommatis* affect the virulence and the symptoms caused by coinfection with pathogens other than *R. rickettsii*. This could reveal if *R. amblyommatis* consistently diminishes the effects of other pathogens in coinfection or if this is only the case with *R. rickettsii*. It is also possible that a previous infection with *R. amblyommatis* could elicit antibodies that diminish the pathogenic effects of other SFG rickettsiae. Current common serological testing available in clinical settings lacks the resolution to detect the different SFG rickettsias, which prevents further understanding of the effects and distribution of *R. amblyommatis* in humans [34,201]. Studying the various strains of *R. amblyommatis* could elucidate if the genetic variability between the different strains leads to differences in the virulence or symptoms caused by each strain. Future studies are required that culture *R. amblyommatis* from different geographic regions and hosts as well as, elucidate the whole genome to characterize different isolates. This may lead to a better understanding of the diversity of *R. amblyommatis* strains, and perhaps their virulence. This research will facilitate the development of more specific pathogen-specific diagnostics.

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Acaricidal Biominerals and Mode-of-Action Studies against Adult Blacklegged Ticks, *Ixodes scapularis*

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Abstract: Ticks in the USA are the most important arthropod vector of microbes that cause human and animal disease. The blacklegged tick, *Ixodes scapularis*, the focus of this study, is able to transmit the bacteria that causes Lyme disease in humans in the USA. The main approach to tick control is the use of chemical acaricides and repellents, but known and potential tick resistance to these chemicals requires the discovery of new methods of control. Volcanic glass, Imergard, was recently developed to mimic the insecticide mode of action of the minerals from diatoms (diatomaceous earth, DE) for the control of malaria mosquitoes in Africa. However, studies on the use of these minerals for tick control are minimal. In a dipping assay, which was put into DE (Celite), the times of 50 and 90% death of adult female *I. scapularis* were 7.3 and 10.5 h, respectively. Our mimic of DE, Imergard, killed ticks in 6.7 and 11.2 h, respectively. In a choice-mortality assay, ticks moved onto a treated surface of Imergard and died at 11.2 and 15.8 h, respectively. Ticks had greater locomotor activity before death when treated by dipping for both Imergard and Celite versus the no-mineral control. The ticks after making contact with Imergard had the mineral covering most of their body surface shown by scanning electron microscopy with evidence of Imergard inside their respiratory system. Although the assumed mode of action of Imergard and Celite is dehydration, the minerals are not hygroscopic, there was no evidence of cuticle damage, and death occurred in as little as 2 h, suggesting minimal abrasive action of the cuticle. Semi-field and field studies are needed in the future to examine the practical use of Imergard and Celite for tick control, and studies need to examine their effect on tick breathing and respiratory retention of water.

Keywords: mechanical insecticides; ticks; Imergard; Celite; repellency

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1. Introduction

Ticks are a nuisance and an important vector of pathogens for humans and animals, including livestock and companion animals. For example, the blacklegged tick, *Ixodes scapularis* Say (Acari: Ixodidae), in the United States, transmits the bacteria, *Borrelia burgdorferi*, that is the causal agent of Lyme disease. Ticks transmit 95% of all vector-borne diseases in the US, with Lyme disease being responsible for greater than 70% of the cases reported [1–3]. *Ixodes scapularis* vectors other pathogens that cause disease, e.g., ehrlichiosis, babesiosis, and anaplasmosis. Chemical pesticides, insecticide-treated clothing, and the application of repellents to skin and clothing are the strategies mostly used to control ticks and to prevent ticks from biting. However, there are concerns about the exposure to pesticides and the growing tick resistance to acaricides [4]. Although resistance has not been found in *I. scapularis* so far, resistance evolution is a possibility [4,5]. Vaccination of animals, such as dogs or rats, which can act as reservoirs of Lyme disease, and cattle, which can be vaccinated against the cattle tick, *Rhipicephalus microplus*, is also used to prevent tick infection [2,6–9]. With an expanding range for ticks that can possibly vector diseases [10,11] and the possibility that being infected with *B. burgdorferi* could increase the survivability of overwintering ticks [12], alternative methods of control are greatly needed.

Mechanical insecticides are minerals, e.g., diatomaceous earth (DE) produced by diatoms, that have a physical mode of action for killing insects. Mechanical insecticides are active against a variety of insects, including some of medical importance: mosquitoes [13,14], flies [15,16], fleas [17], bedbugs [18], beetles [19–22], cockroaches [23], and thrips [24], and they could represent a new method for tick control without the concerns associated with chemical pesticides. The hypothesized mode of action of mechanical insecticides is abrasion of the cuticle through a physical action or through the removal of cuticular lipids by absorption, which causes dehydration and death [16,17].

Most recently, the industrial minerals diatomaceous earth (Celite 610) and a newly discovered mechanical insecticide, Imergard™, were shown to be active against larval and nymphal lone star ticks, *Amblyomma americanum* [25,26]. Imergard was originally developed from volcanic glass for adult mosquito control, as a residual wall spray for use in homes in Africa [13,14]. Richardson et al. [27] found Celite and Imergard to be highly efficacious against larval blacklegged ticks. Showler and Harlien [28] found that Celite could be used to prevent larval southern cattle fever ticks, *R. microplus*, from attaching to and feeding on calves. Although these minerals were active against three different species of larval ticks, they were not tested against adult ticks. Also, their mode of action in ticks is not well studied. Richardson et al. [27] provided evidence that the minerals might block the spiracles of nymphal blacklegged ticks.

The current study examined the efficacy of Celite and Imergard against adult female *I. scapularis* by two exposure methods, dipping and a choice-mortality assay. In addition, mode-of-action studies were conducted by examining the locomotor activity after treatment and the positioning of the minerals on the tick surface, including the openings into the respiratory system (by scanning electron microscopy).

2. Materials and Methods

2.1. Mechanical Insecticides and Ticks

Unfed adult female blacklegged ticks, *I. scapularis*, were purchased from Oklahoma State University (Oklahoma City, OK, USA). The ticks were approximately 70–90 days post-molt and were used within two weeks of arrival. Until used, the ticks were maintained at 27 ± 1 °C and 70 ± 5% relative humidity with a 14:10 L:D cycle.

The mechanical insecticides Imergard™ WP and Celite 610 were obtained from the company Imerys Filtration Minerals, Inc. (Roswell, GA, USA) and stored at room temperature in the laboratory in their original packaging until needed. All bioassays were conducted in a Percival I-36NL incubator (Percival Scientific, Inc., Perry, IA, USA) under environmental conditions described later.

2.2. Dipping Assay

Ticks were dry-dipped for 1 s in 25 mg of mechanical insecticide, either Celite or Imergard, in the bottom of a 60 mm × 15 mm plastic Petri dish (Fisher Scientific, Hampton, NH, USA) using a round #4 camelhair brush (Craft Smart, Irving, TX, USA). Each tick on the tip of the brush was completely submerged below the surface of the powder. After dipping, the tick was placed into a clean 60 mm × 15 mm plastic Petri dish (one tick per dish), and the dish bottom and top were immediately sealed together along the outer edge with Parafilm (Bemis Company, Neenah, WI, USA). After each dipping, the camelhair brush was cleaned by brushing the surface of a 9 cm-diameter, coarse-grade filter paper (Fischerbrand, Walthem, MA, USA) on a flat benchtop. A new dish containing 25 mg of mechanical insecticide was replaced after treating 10 ticks. For the controls, each undipped tick was transferred with a camelhair brush (that never came in contact with the mechanical insecticides) into a clean Petri dish, and the Petri dish was processed as described for the treatments. The dishes containing treated and control ticks were then incubated at 30 ± 1 °C, 50 ± 5% relative humidity, and during the photophase. These conditions were used so the results would be directly comparable to our previous results with mosquitoes [13] and filth flies [15]. In this study, the ticks were observed every 30 min until all ticks were dead. Death was defined as

no tick movement after gently tapping the center of the Petri dish. The treatments and controls were each replicated three times (10 ticks per replicate), and mortality data were analyzed by repetitive sampling (observation of death) every 30 min using a Probit model (described later).

2.3. Tick Movement

At 1 h intervals for both the treatments and the controls in the dip bioassays for both Celite and Imergard, the position of the tick on the Petri dish bottom was marked in ink by consecutive numbers on the outside top of the Petri dish. After the ticks were all dead, the distance each tick moved between each mark was measured ±1 mm for each 1 h interval up to the LT_{50} (the time to 50% mortality) and used as an index of tick locomotor activity. Before the LT_{50}, any time intervals with no tick movement, the tick was considered dead; this zero movement was not recorded, but the earlier distance moved was included in the analysis.

2.4. Choice-Mortality Assay

This assay was conducted in 12 cm × 3.5 cm glass Petri dishes (Fisher Scientific). Scotch blue tape #2090 (3M, St. Paul, MN, USA) was applied to half of the Petri dish bottom (sticky surface to the glass), and 2 g of Imergard was added to the center of the Petri plate bottom and was spread as evenly as possible by sight over the entire surface of the bottom using a metal spatula. The tape with Imergard on top was removed slowly to prevent any transfer of the mineral to the Petri dish glass bottom under the tape. This action produced a plate where half of the bottom contained Imergard, and the other half was Imergard free.

One tick was placed approximately in the center of each dish but on the untreated surface, using a clean camelhair brush (previously described). Then, the top of the Petri dish was applied, and the dish sealed with Parafilm as described earlier. The controls were conducted the same except the Petri dish did not contain Imergard. The dishes containing treated and control ticks were then incubated at 30 ± 1 °C, 50 ± 5% relative humidity and during the photophase except for the last three observations, and the ticks observed every h until all ticks were dead. For the observations during the scotophase, the incubator lights were briefly turned on to score mortality. Death was defined as no tick movement after gently tapping the center of the Petri dish. Five dishes were used per replicate and three replicates were conducted for both the treatments and control. Mortality data were analyzed by repetitive sampling (observations of death) every 60 min using a Probit model (described later).

2.5. Scanning Electron Microscopy (SEM)

Ticks from the choice-mortality assay were stored individually at −40 °C until needed for SEM analysis. The SEM analysis was conducted at the Analytical Instrumentation Facility on the North Carolina State University Centennial Campus (Raleigh, NC, USA). The ticks were desiccated in a vacuum for 48 h and then fixed with super glue onto a Hitachi SEM aluminum mount. The samples were subjected to Cressington sputter coating for 60 s with a 70 nm gold–palladium mixture (60 Au/40 Pd). A Hitachi SU3900 (Hitachi, Ltd., Chiyoda City, Tokyo, Japan) variable pressure scanning electron microscope was used to image the tick surface for both treatments and the control.

2.6. Statistical Analysis

Mortality data were collated using Excel 2016 (Microsoft Co, Redmond, WA, USA). Probit, ANOVA, and t-tests were performed in JMP Pro 16 (JMP Statistical Discovery LLC, Cary, NC, USA). Abbott's correction was utilized when necessary, but only one control tick died in the entire study.

3. Results

3.1. Mortality of Dipped I. scapularis Adults

Figure 1 shows percentage mortality versus time after treatment for adult female *I. scapularis* dipped into Celite (Figure 1A) or Imergard (Figure 1B). For Celite, the first mortality occurred at 3.5 h with 100% mortality obtained after 12 h (Figure 1A). There was no mortality in the controls for the first 10.5 h, and one tick died at 11 h. The data shown in Figure 1A is not Abbot corrected. The probit model (Abbot corrected) for these data are shown in Table 1. The LT_{50} (the time to 50% mortality) and LT_{90} (the time to 90% mortality) for Celite were 7.29 and 10.5 h, respectively, and were statistically significantly different (no overlap of the 95% confidence intervals).

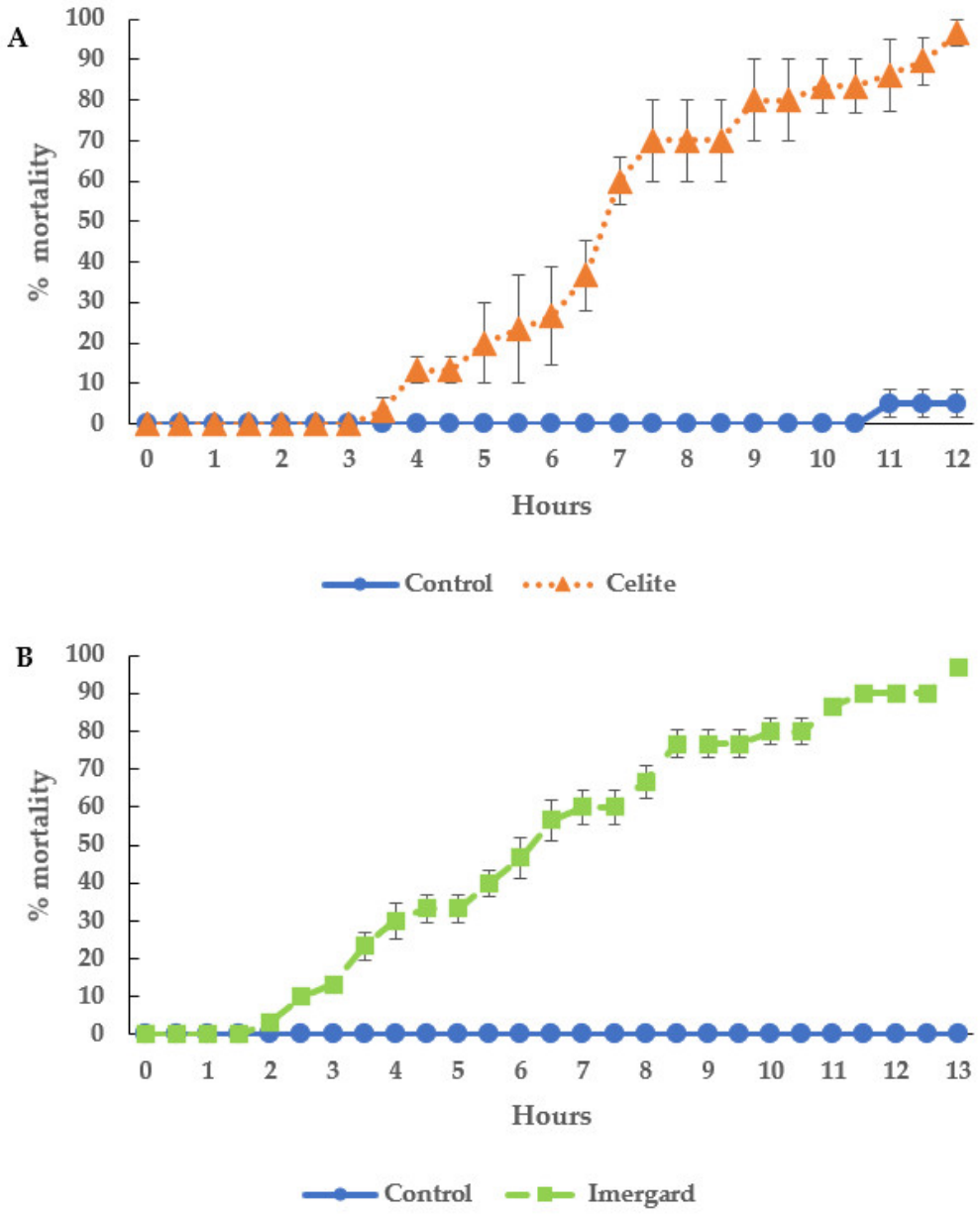


Figure 1. Mortality of unfed adult female *Ixodes scapularis* dipped in (**A**) Celite and (**B**) Imergard and then incubated in the photophase at 30 ± 1 °C and 50 ± 5% relative humidity. Error bars are ±1 SEM. In some cases, the error bars were too small to be visible.

Table 1. Probit models for *Ixodes scapularis* in the dipping and choice-mortality assays †.

Mechanical Insecticide	N	Slope (SEM)	LT_{50} † (95% CL)	LT_{90} † (95% CL)	X^2
Celite 610 dip	30	0.399 (0.0246)	7.29 A ‡ (6.98–7.60)	10.5 A ‡ (10.0–11.1)	17.56
Imergard dip	30	0.358 (0.0310)	6.72 A (6.35–7.09)	11.2 A (10.6–11.9)	8.90
Imergard choice-mortality	15	0.280 (0.0321)	11.2 B (10.5–12.0)	15.8 B (14.6–17.5)	2.32

† LT_{50} and LT_{90} is the time in h to 50 and 90% mortality, respectively (CL = confidence limit; X^2 = chi-square). ‡ Values in the same column with overlapping 95% CLs are considered not statistically significant and are designated by the same letter.

Imergard killed the ticks in a similar time to Celite. The first mortality occurred at 2 h, and all of the ticks were dead after 17 h, with no mortality in the control (Figure 1B). The LT_{50} and LT_{90} for Imergard were 6.72 and 11.2 h, respectively, and were statistically significantly different (no overlap of the 95% confidence intervals) (Table 1). At the LT_{50} and LT_{90}, there was no statistical differences between Celite and Imergard (Table 1).

3.2. Mortality of *I. scapularis* Adults in the Choice Mortality Assay

The ticks remained on the Petri dish bottom for the entire assay, started dying at 6 h, and were all dead after 17 h (Figure 2, no mortality in the control), which was longer than for Celite and Imergard in the dipping assay (Figures 1A and 1B, respectively). The LT_{50} and LT_{90} for the choice-mortality assay were 11.2 and 15.8 h, respectively, which were statistically significantly different (no overlap of the 95% confidence intervals (Table 1)). The LT_{50} and LT_{90} were also longer than that for Celite and Imergard in the dipping assay (Table 1). In the choice-mortality assay, the LT_{50} and LT_{90} for Imergard were 1.66- and 1.41-fold, respectively, longer than that for Imergard in the dipping assay (Table 1).

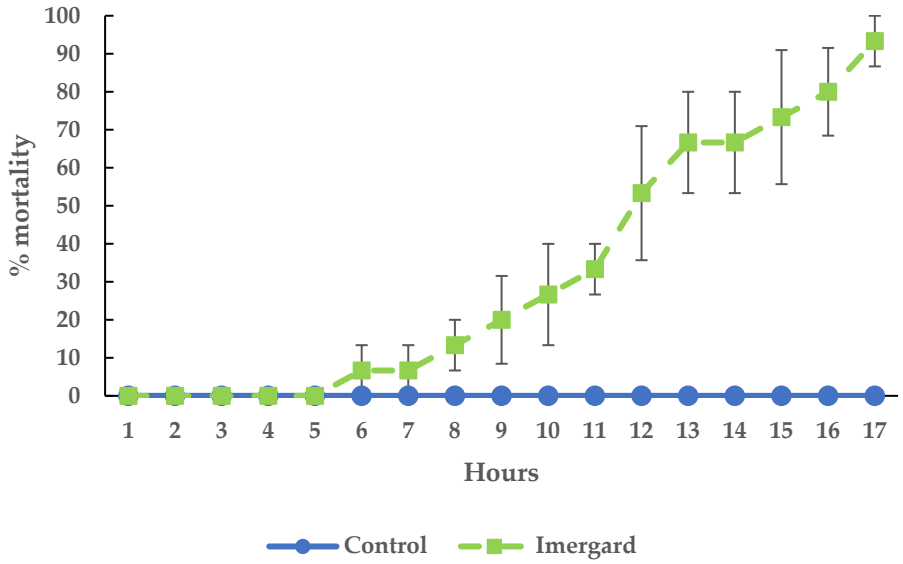


Figure 2. Mortality of *Ixodes scapularis* in the choice-mortality assay. The assay was conducted at 30 ± 1 °C and 50 ± 5% relative humidity during the photophase except for the last 3 h. Error bars are ±1 SEM. In some cases, the error bars were too small to be visible.

3.3. Mineral Exposure by Dipping on *I. scapularis* Movement

The location of the ticks in the Petri dish after dipping into Celite and Imergard was marked on the dish lid every hour, and the distance between each location was measured for each time interval as an index of the locomotor activity for the treatment compared to the control. The results for Celite for 1–7 h are shown in Figure 3A; data collection was stopped at the LT_{50} (Table 1). Distance moved at 2–4 h for the treatment was higher than the control (ANOVA, F = 7.18, df = 117, p > F = 0.0084), but no differences were noted at 5–7 h (ANOVA, F = 0.151, df = 101, p > F = 0.699). If you compare the average movement of

the treatment versus the control at 2–4 h (Figure 3B), treatment movement was also higher (*t*-test, $t(115) = 0.0084$, $p = 0.05$) than the control, while this was not the case at 5–7 h (*t*-test, $t(99) = 0.6888$, $p = 0.05$). This increased movement over the control was also found for Imergard but at all time points after 1 h through 5 h (Figure 3C, ANOVA, F = 10.4, df = 141, $p > F = 0.0016$; Figure 3D, *t*-test, $t(137) = 0.0014$, $p = 0.05$). Data collection was stopped at the LT_{50} (Table 1).

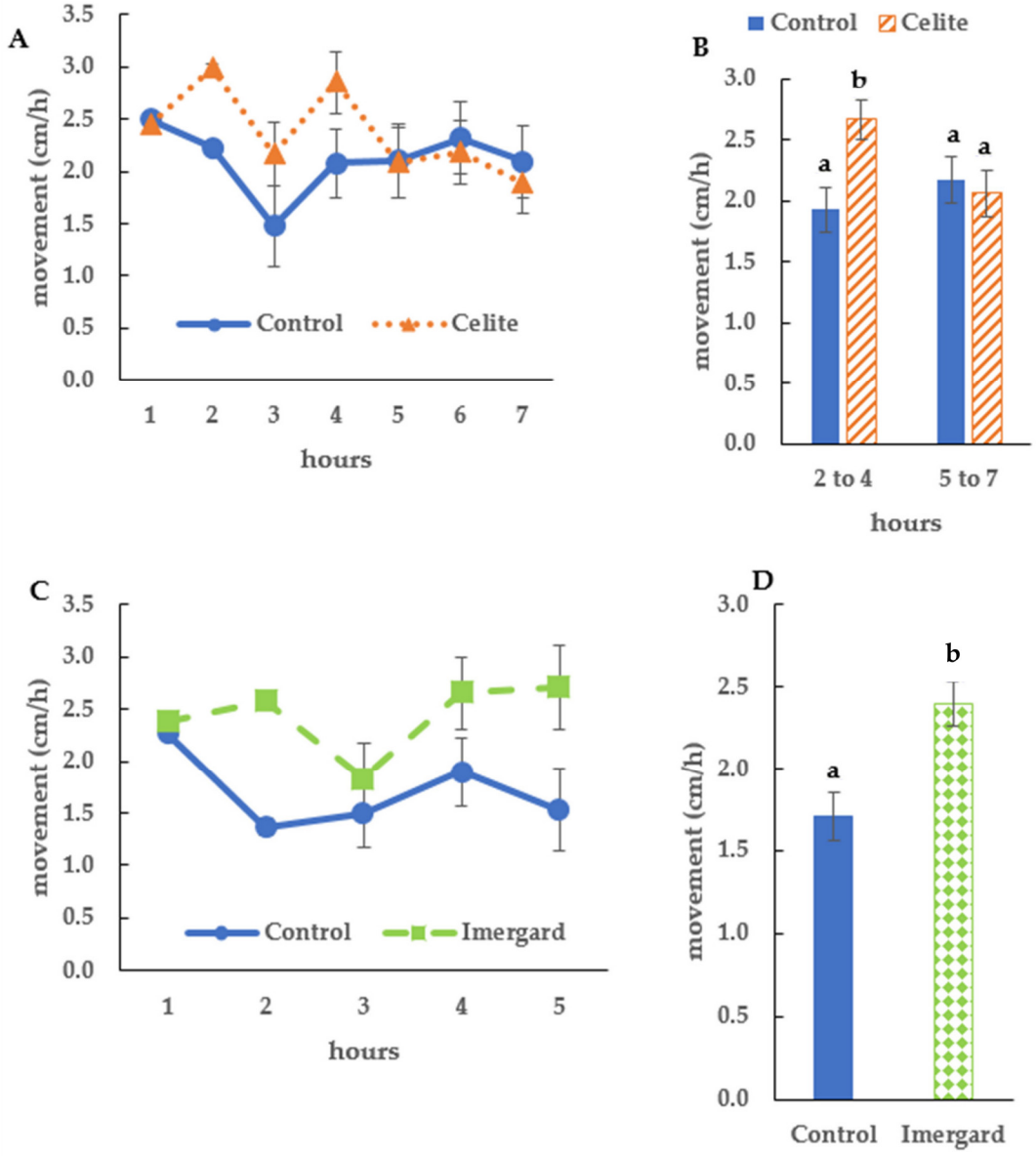


Figure 3. (**A**) Movement of Celite dipped versus control ticks, (**B**) the average distance traveled of Celite dipped versus control ticks between two periods of movement from 2–4 and 5–7 h, (**C**) movement of Imergard dipped versus control ticks from 2–5 h, and (**D**) the average distance traveled of Imergard dipped versus control ticks during 2–5 h. In (**B**,**D**), different lower case letters indicated a statistically significant difference as determined by a *t*-test (see results for details). Error bars are ±1 SEM. In some cases, the error bars were too small to be visible.

3.4. Scanning Electron Microscopy of I. scapularis Exposed to Imergard in the Choice Mortality Assay

Figure 4 shows a close up by SEM of Imergard on the tick surface. The mineral is essentially amorphous, with no regular shape. Scanning electron microscopy of Imergard

on different parts of the tick body are shown in Figure 5B,D,F compared to untreated ticks (Figure 5A,C,E). The pictures show an even coating on the cuticle with some major clumping present (shown with arrows in Figure 5B,D,F). No evidence of cuticular damage was observed. The spiracular plate and aeropyles (Figures 6A and 6C, respectively) show the normal state of the entrance to the tick tracheal system in the control. In treated ticks, the plate is covered with Imergard (Figure 6B,D), and in some cases Imergard had penetrated into the aeropyle (Figure 6D; far left arrow).

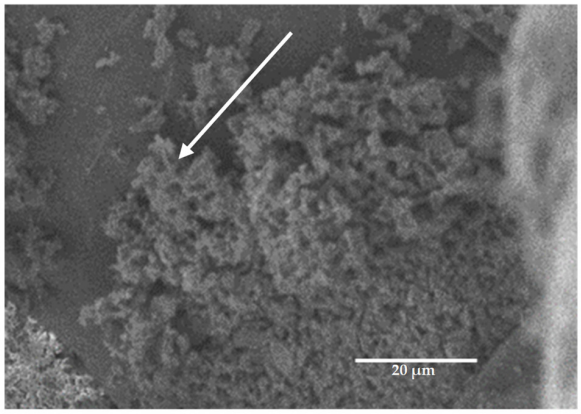


Figure 4. Close view by scanning electrosn microscopy of Imergard (arrow) on the surface of *Ixodes scapularis*.

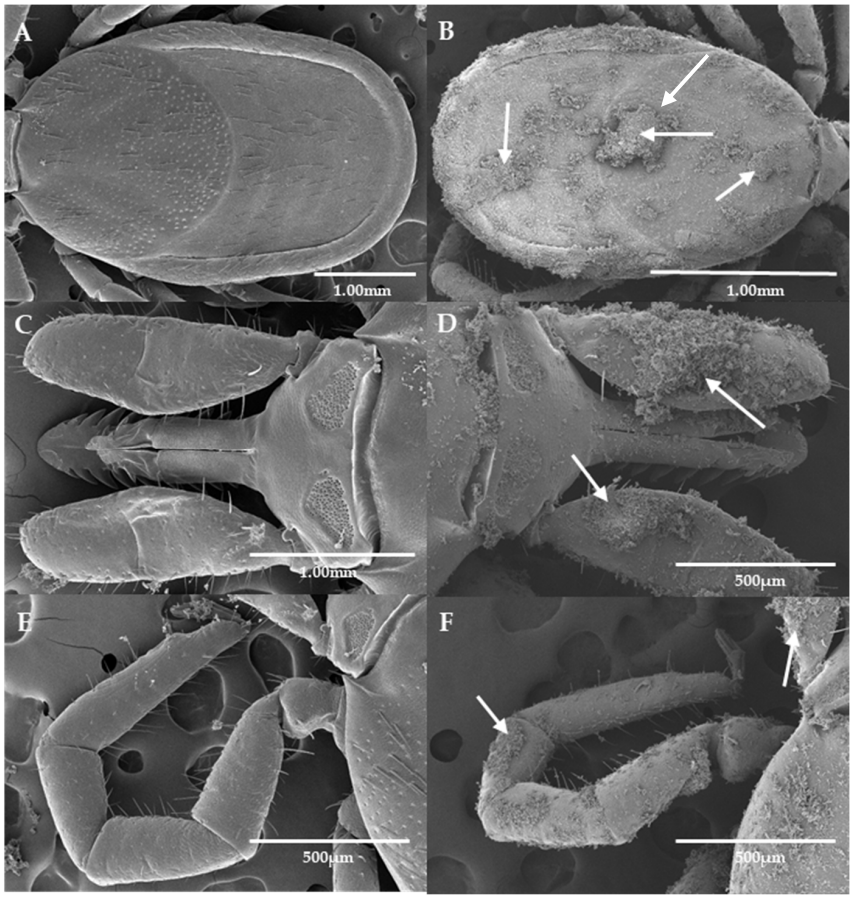


Figure 5. Scanning electron microscopy of control (**left**) and treated (**right**) *Ixodes scapularis* in the Imergard choice-mortality assay. (**A**,**B**) full body, (**C**,**D**) capitulum, and (**E**,**F**) leg. Arrows show clumping of Imergard. The reason for clumping is unknown.

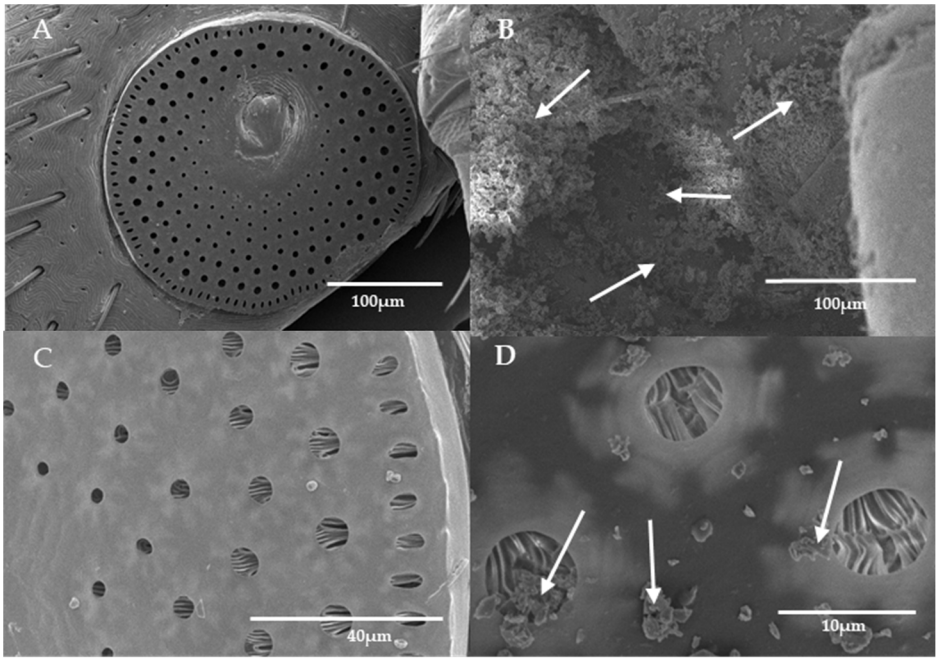


Figure 6. Scanning electron microscopy of the (**A**) control spiracular plate (round structure with holes), (**B**) treated spiracular plate, (**C**) control aeropyles (holes in the spiracular plate), and (**D**) treated aeropyles of *Ixodes scapularis* in the Imergard choice-mortality assay. Arrows in (**B**) show Imergard blockage of the openings in the spiracular plate. Arrows in (**D**) show Imergard inside of an aeropyle (far left arrow) or otherwise on the surface of the spiracular plate.

4. Discussion

Two mechanical insecticides, Celite 610 and Imergard WP, were investigated for their acaricidal activity against unfed, adult, female blacklegged ticks, *I. scapularis.* It is well established that minerals produced by some diatoms like that found in Celite (diatomaceous earth, DE) are insecticidal, with far less information about their use to control ticks [29]. A few years ago we developed an alternative to diatomaceous earth, made from volcanic glass, Imergard, as a residual wall spray for mosquito control inside of homes in Africa [13,14]. The advantages of Imergard, it does not contain crystalline silica found in low levels in diatomaceous earth, and volcanic glass is highly abundant. Imergard easily suspends in water, can be applied by spraying using typical equipment used for vector control, and controlled mosquitoes for at least 6 months in phase II field trials in Benin, Africa [14].

In a simple dipping assay, we found 50 and 90% of adult female *I. scapularis* ticks were dead in 6.72 and 11.2 h, respectively, when exposed to Imergard; the time to death for the mineral, Celite (diatomaceous earth), was 7.29 and 10.5 h, respectively, not significantly statistically different from Imergard (Table 1). Showler et al. [25] and Showler and Harlien [26] found Celite and Imergard were able to control larval and nymphal lone star ticks, *A. americanum.* Richardson et al. [27] found Celite and Imergard were highly efficacious against nymphal blacklegged ticks. Showler and Harlien [28] found that Celite could be used to prevent larval southern cattle fever ticks, *R. microplus*, from attaching and feeding on calves. Although these minerals were active against three different species of ticks in the above published studies, they were not tested against adult ticks. The current study is the first evidence that Celite and Imergard could control unfed, female adult blacklegged ticks.

Richardson et al. [27] used the same dipping assay, tick age after molting (50–70 days) and environmental conditions post treatment with Imergard and Celite on unfed nymphal, blacklegged ticks (mixed sexes) as that used in the current study for adult ticks. Comparing the different stages, adult ticks appear to be less susceptible than nymphal ticks, both about the same age after molting. For example, the LT_{50} and LT_{90} were 5.4- and 4.9-fold, respectively, longer for adult females than nymphs for Celite and 5.7- and 6.0-fold,

respectively, longer than nymphal ticks for Imergard. Richardson et al. [27] also found Imergard was slightly more effective (had a shorter LT_{50} and LT_{90}) than Celite in nymphal *I. scapularis* but this was not the case for adult females of the same species (Table 1). The mode of action of mechanical insecticides like Celite is thought to be through physically damaging the cuticle, resulting in water loss [16,17], that upsets osmotic balance and finally causes death. This could occur by the abrasive action of the mineral disrupting the outer water proofing wax layer of the cuticle as body parts rub against each other and/or by rubbing against solid substrates as the insect moves; and/or the mineral absorbing the wax layer of the cuticle. Deguenon et al. [13,14] suggested the mechanism of abrasion was not likely in mosquitoes for Imergard. When they landed on an Imergard treated surface, the mineral transferred mostly to the lower legs, presumably by electrostatic charge differences, the insect quickly became quiescent, and then the mosquito died demonstrating symptoms similar to pyrethroid poisoning.

Chen et al. [15] found that for three species of filth flies exposed in a modified WHO cone test to a surface treated with Imergard, the entire body was covered with minerals, completely different from that for mosquitoes [13]. The flies also were more active after exposure unlike mosquitoes. More research is needed to better quantify this increased fly activity. Adult *I. scapularis* females were similar to flies in two ways. The choice-mortality assay showed their entire bodies were covered with mineral (Figure 5), and after the dipping-mortality assay, the index for adult movement increased compared to controls (Figure 3). This increased movement could be the result of the mineral being an irritation to the ticks, and movement is a method to reduce this irritation. Richardson et al. [27] also found blacklegged nymphs dipped into Celite had all of their body surfaces covered with the mineral; however, activity levels after dipping were not measured. In the case of nymphs, they started dying in 10–20 min, suggesting abrasion was not a factor in this short time frame and where tick overall movement appears by observation to be significantly less than filth flies. Increased activity levels in ticks and filth flies after treatments do suggest they are able to detect the mineral on their body surface by some method that is not currently known. In the case of flies, the increase in activity occurred in seconds after mineral application, while for adult, female *I. scapularis*, increased movement was detected after 1 h (Figure 3).

In the Chen et al. [15] filth fly studies, Imergard killed the smaller blow flies and house flies faster than the larger flesh flies. The same relationship between weight and time to death was found if you compare the time to death for smaller blacklegged nymphal ticks [27] to that for larger adult female *I. scapularis*; the fold differences for Imergard and Celite were mentioned earlier in this discussion. Volume increases by the cube, surface area by the square as the size of an insect or tick increases (where the body shape is similar). The surface/volume ratio of the adult female tick is smaller relatively to that for nymphs. The hypothesis would be that a topically active pesticide that causes dehydration would have a greater effect for nymphs as our research showed. Dawson [30] also reported that diatomaceous earth was more active for a smaller body size.

Richardson et al. [27] found that the time to death for older unfed *I. scapularis* nymphs was longer than younger unfed nymphs and hypothesized that this resulted from more mature cuticle with greater protection from water loss by an enhanced wax layer as one possibility. This result with older nymphs was unexpected since these ticks do not consume water before blood feeding. The older ticks would be expected to be more dehydrated and more susceptible to a mechanical insecticide that disrupts the water barrier of the cuticle. Although we have no data to support this hypothesis, there may be some evidence to support this idea between unfed nymphal and adult ticks. Yoder et al. [31] found 3.6-fold greater cuticular lipid in 30 d post-molt, adult versus nymphal lone star ticks, *A. americanum*. If this increase in the wax layer occurs between nymphal and adult *I. scapularis* as well, this might explain the longer time to death after dipping into Celite and Imergard for the blacklegged tick adult. There are also fundamental differences in adult female cuticle

compared to nymphs, where the former must be prepared to increase their body size several fold when feeding to repletion.

Richardson et al. [27], in dipping-mortality assays, found that, in some cases, the blacklegged nymphal tick spiracular sieve plate was heavily blocked with Celite. In the current study with adult female *I. scapularis* in the choice-mortality assay, the plate was also covered with Imergard at high levels in some cases (Figure 6B). Since the sieve plate was visible in some treatments, we were able to view the aeropyles and found Imergard inside of the small openings into the respiratory system (Figure 6D). This could have also been the case for Celite in the studies by Richardson et al. [27], where it was hypothesized that the rapid death of ticks in 20 min or less might partly be a result of blockage of the respiratory system.

Finally, the choice-mortality assay for *I. scapularis* female adults is encouraging, suggesting Imergard could be used as a residual treatment for tick control not requiring direct contact with the tick at the time of application. If Imergard had any repellency, it was not enough to prevent them from walking onto a treated surface in our choice-mortality assay and obtaining a lethal dose. These results are consistent with those of Deguenon et al. [13] that found Imergard treated surfaces were not repellent to mosquitoes or Chen et al. [15] which found Imergard was not repellent to three species of filth flies to the point of preventing the acquisition of a lethal dose. Semi-field and field studies are needed in the future to determine the practical use of Imergard and Celite for the control of the blacklegged tick and other tick species. Showler and Harlien [28] found that Celite could be used to prevent larval southern cattle fever ticks, *R. microplus*, from attaching and feeding on calves, providing additional evidence of the practical use of these mechanical insecticides for tick control.

5. Conclusions

In conclusion, both Celite 610 and Imergard WP in dipping assays were lethal to adult, unfed, female *I. scapularis* ticks in the laboratory. The mechanical insecticide Imergard in choice-mortality assays was also lethal, demonstrating that the mineral repellency, if any, was not enough to prevent them from walking onto a treated surface and receiving a lethal dose. Celite and Imergard treatments increased tick locomotor activity. Ticks also, after walking onto a treated surface of Imergard, had their entire bodies covered with the mineral with evidence of mineral intrusion into the sieve-plate aeropyles, the entrance to their respiratory system. Further semi-field and field studies are needed to determine the practical use of these minerals in the control of the Lyme disease tick and other species.

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Data Availability Statement: Data available upon justifiable request by the corresponding author.

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(54) **INSECT AND TICK REPELLENT FORMULATIONS AND METHODS OF USE THEREOF**

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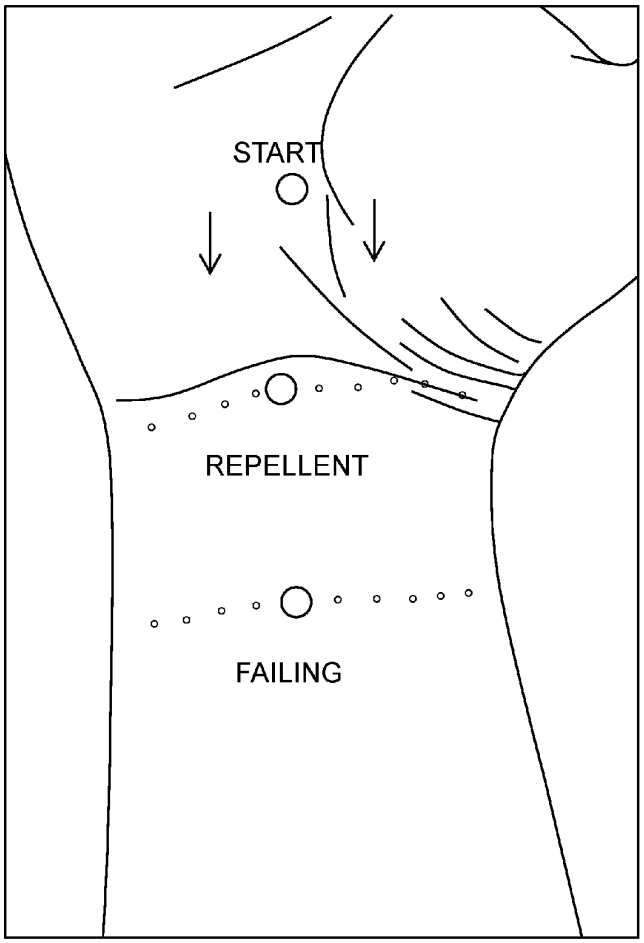
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(57) **ABSTRACT**

The invention relates to a formulation comprising: 2-undecanone in an amount of about 5% to about 50%; at least one hydrophobic base in an amount of about 5% to about 65%; at least one alcohol in an amount of about 5% to about 50%; and at least one emulsifier in an amount of about 5% to about 15% and methods of repelling insects and ticks by applying to a subject or a substrate a formulation of the invention.



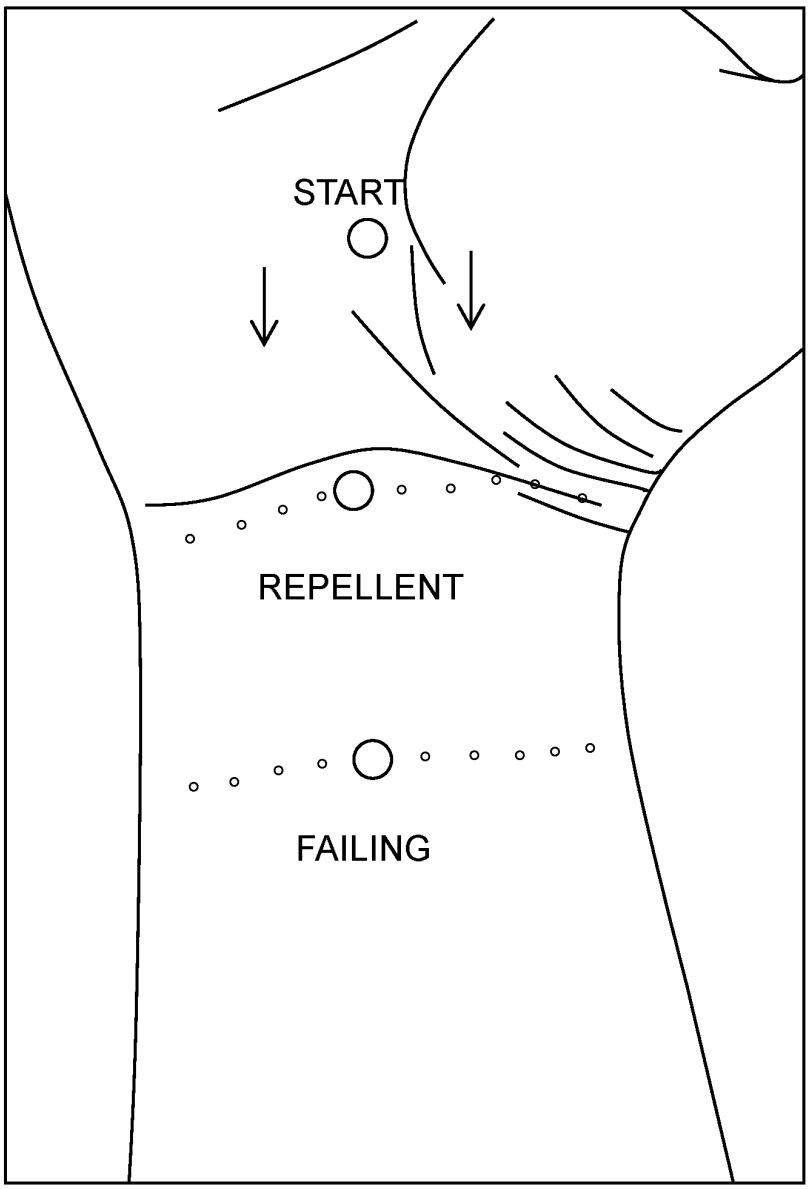


FIG. 1

INSECT AND TICK REPELLENT FORMULATIONS AND METHODS OF USE THEREOF

STATEMENT OF PRIORITY

[0001] This application is a continuation application of U.S. patent application Ser. No. 16/958,102, filed on Jun. 25, 2020, a 35 U.S.C. § 371 national phase application of International Application Serial No. PCT/US2018/067625, filed Dec. 27, 2018, which claims the benefit, under 35 U.S.C. § 119 (e), of U.S. Provisional Application No. 62/611,178 filed on Dec. 28, 2017, the entire contents of each of which is incorporated by reference herein.

FIELD OF THE INVENTION

[0002] The present invention concerns methods and formulations for repelling insects and ticks.

BACKGROUND OF THE INVENTION

[0003] Insect and tick repellants are widely used throughout the United States and throughout the world. In some regions, the use of repellants is important for avoiding or reducing the occurrence of diseases carried by insects and ticks. For example, the Centers for Disease Control (CDC) receives nearly 10,000 reports of Lyme disease (transmitted by deer ticks) and 1,000 reports of encephalitis (transmitted by mosquitoes) annually.

[0004] Currently, the most common repellent is N,N-diethyl-meta-toluamide (DEET). DEET was designed to be applicable to the skin of subjects, and is designed to repel rather than kill insects and ticks. Although in use for some time, concern has recently emerged about the potential toxicity of DEET to children. Recently the US Environmental Protection Agency (EPA) determined that it would no longer allow child safety claims on labels for DEET-containing products.

[0005] Improved insect and tick repellants useful for all persons are needed.

BRIEF DESCRIPTION OF THE DRAWINGS

[0006] FIG. **1** provides photograph of a representative arm with descriptive labels to show the set-up for testing tick foraging. The labels were not present during actual testing.

SUMMARY OF THE INVENTION

[0007] A first aspect of the invention provides a formulation comprising: 2-undecanone in an amount of about 5% to about 50% by volume; at least one hydrophobic base in an amount of about 5% to about 65% by volume; at least one alcohol in an amount of about 5% to about 50% by volume; and at least one emulsifier in an amount of about 5% to about 15% by volume.

[0008] A second aspect provides a formulation comprising: 2-undecanone in an amount of about 5% to about 30% by volume; at least one hydrophobic base in an amount of about 25% to about 40% by volume; at least one alcohol in an amount of about 25% to about 40% by volume; and at least one emulsifier in an amount of about 5% to about 15% by volume.

[0009] A third aspect of the invention provides a formulation comprising: 2-undecanone in an amount of about 10% to about 25% by volume; at least one hydrophobic base in an amount of about 25% to about 35% by volume; at least one alcohol in an amount of about 25% to about 40% by volume; and at least one emulsifier in an amount of about 5% to about 15% by volume.

[0010] A fourth aspect of the invention provides a method of repelling an insect pest or a tick, comprising: applying to a subject a formulation of the present invention in an amount effective to repel the insect or tick.

DETAILED DESCRIPTION

[0011] The present invention now will be described hereinafter with reference to the accompanying drawings and examples, in which embodiments of the invention are shown. This description is not intended to be a detailed catalog of all the different ways in which the invention may be implemented, or all the features that may be added to the instant invention. For example, features illustrated with respect to one embodiment may be incorporated into other embodiments, and features illustrated with respect to a particular embodiment may be deleted from that embodiment. Thus, the invention contemplates that in some embodiments of the invention, any feature or combination of features set forth herein can be excluded or omitted. In addition, numerous variations and additions to the various embodiments suggested herein will be apparent to those skilled in the art in light of the instant disclosure, which do not depart from the instant invention. Hence, the following descriptions are intended to illustrate some particular embodiments of the invention, and not to exhaustively specify all permutations, combinations and variations thereof.

[0012] Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. The terminology used in the description of the invention herein is for the purpose of describing particular embodiments only and is not intended to be limiting of the invention.

[0013] All publications, patent applications, patents and other references cited herein are incorporated by reference in their entireties for the teachings relevant to the sentence and/or paragraph in which the reference is presented.

[0014] Unless the context indicates otherwise, it is specifically intended that the various features of the invention described herein can be used in any combination. Moreover, the present invention also contemplates that in some embodiments of the invention, any feature or combination of features set forth herein can be excluded or omitted. To illustrate, if the specification states that a composition comprises components A, B and C, it is specifically intended that any of A, B or C, or a combination thereof, can be omitted and disclaimed singularly or in any combination.

[0015] As used in the description of the invention and the appended claims, the singular forms "a," "an" and "the" are intended to include the plural forms as well, unless the context clearly indicates otherwise.

[0016] Also, as used herein, "and/or" refers to and encompasses any and all possible combinations of one or more of the associated listed items, as well as the lack of combinations when interpreted in the alternative ("or").

[0017] The term "about," as used herein when referring to a measurable value such as a dosage or time period and the like refers to variations of ±10%, ±5%, ±1%, ±0.5%, or even ±0.1% of the specified amount. As used herein, phrases such

as "between X and Y" and "between about X and Y" should be interpreted to include X and Y. As used herein, phrases such as "between about X and Y" mean "between about X and about Y" and phrases such as "from about X to Y" mean "from about X to about Y."

[0018] The term "comprise," "comprises" and "comprising" as used herein, specify the presence of the stated features, integers, steps, operations, elements, and/or components, but do not preclude the presence or addition of one or more other features, integers, steps, operations, elements, components, and/or groups thereof.

[0019] As used herein, the transitional phrase "consisting essentially of" means that the scope of a claim is to be interpreted to encompass the specified materials or steps recited in the claim and those that do not materially affect the basic and novel characteristic(s) of the claimed invention. Thus, the term "consisting essentially of" when used in a claim of this invention is not intended to be interpreted to be equivalent to "comprising."

[0020] As used herein, the terms "increase," "increasing," "increased," "enhance," "enhanced," "enhancing," and "enhancement" (and grammatical variations thereof) describe an elevation of at least about 25%, 50%, 75%, 100%, 150%, 200%, 300%, 400%, 500% or more as compared to a control.

[0021] As used herein, the terms "reduce," "reduced," "reducing," "reduction," "diminish," and "decrease" (and grammatical variations thereof), describe, for example, a decrease of at least about 5%, 10%, 15%, 20%, 25%, 35%, 50%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 100% as compared to a control. In particular embodiments, the reduction can result in no or essentially no (i.e., an insignificant amount, e.g., less than about 10% or even 5%) detectable activity or amount.

[0022] The present invention provides monophasic formulations comprising undecanone for use in repelling insects and/or ticks. Thus, in some embodiments, the formulations of the invention are not in the form of emulsions but are in a single phase.

[0023] In some embodiments, the formulations may be in the form of an emulsion that is homogeneous.

[0024] In some embodiments, the formulations of the invention may be used to repel animals generally including, but not limited to, insects, ticks, spiders, hunting spiders, and scorpions.

[0025] Notably, 2-undecanone alone is not an effective insect or tick repellent for at least the reasons that it evaporates too fast and its duration of repellency is less than about 30 min. The formulations of the present invention comprising 2-undecanone, at least one hydrophobic base (for example, a seed oil), at least one alcohol, and at least one emulsifier provide significant repellency lasting over many hours after application to a subject.

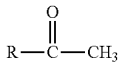
[0026] Accordingly, in some embodiments, the present invention provides a formulation comprising: 2-undecanone in an amount of about 5% to about 50% by volume (e.g., about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 50%, or any range or value therein); at least one hydrophobic base in an amount of about 5% to about 65% by volume (e.g., about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 50%, 55%, 60%, 65%, or any range or value therein); at least one alcohol in an amount of about 5% to about 50% by volume (e.g., about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 50%, or any range or value therein); and at least one emulsifier (i.e., total amount of all emulsifiers) in an amount of about 5% to about 15% by volume (e.g., about 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, or any range or value therein). In some embodiments, the total amount of hydrophobic base in the formulation may be in a range from about 5%-65% by volume of the formulation. In some embodiments, the total amount of alcohol in the formulation may be in a range from about 5% to about 50% by volume of the formulation. In some embodiments, the total amount of emulsifier in the formulation may be in a range from about 5% to about 15% by volume of the formulation.

[0027] In some embodiments, a formulation is provided comprising: 2-undecanone in an amount of about 5% to about 30% by volume (e.g., about 5%, 10%, 15%, 20%, 25%, 30%, or any range or value therein); at least one hydrophobic base in an amount of about 25% to about 40% by volume (e.g., about 25%, 26%, 27%, 28%, 29%, 30%, 13%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, or any range or value therein); at least one alcohol in an amount of about 25% to about 40% by volume (e.g., about 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40% by volume, or any range or value therein); and at least one emulsifier (i.e., total amount of all emulsifiers) in an amount of about 5% to about 15% by volume or about 5% to about 15% by volume (e.g., about 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, or any range or value therein). In some embodiments, the total amount of hydrophobic base in the formulation may be in a range from about 25%-40% by volume of the formulation. In some embodiments, the total amount of alcohol in the formulation may be in a range from about 25% to about 40% by volume of the formulation. In some embodiments, the total amount of emulsifier in the formulation may be in a range from about 5% to about 15% by volume of the formulation.

[0028] In some embodiments, the present invention provides a formulation comprising: 2-undecanone in an amount of about 10% to about 25% by volume (e.g., about 10%, 15%, 20%, 21%, 22%, 23%, 24%, 25%, or any range or value therein; e.g., about 12% to about 16%, about 12% to about 17%, about 13% to about 16%, about 13% to about 17%, about 14% to about 16% by volume, or any range or value therein); at least one hydrophobic base in an amount of about 25% to about 35% by volume (e.g., about 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35%, or any range or value therein; e.g., about 26% to about 33%, about 26% to about 35%, about 28% to about 32%, about 28% to about 33%, about 28% to about 35%,%, or any range or value therein); at least one alcohol in an amount of about 25% to about 40% by volume (e.g., about 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, or any range or value therein; e.g., about 30% to about 38%, about 30% to about 40%, about 32% to about 37%, about 32% to about 38%, about 33% to about 37%, about 33% to about 36%, or any range or value therein); and at least one emulsifier (i.e., total amount of all emulsifiers) in an amount of about 5% to about 15% by volume or about 5% to about 15% by volume (e.g., about 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, or any range or value therein; e.g., about 7% to about 12%, about 7% to about 13%, about 7% to about 14%, about 8% to about 11%, about 8% to about 12%, about 8% to about 13%, or any range or value therein). In some embodiments, a formulation is provided comprising 2-undecanone in an

amount of about 10% to about 20% by volume (e.g., about 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, or any range or value therein); at least one hydrophobic base in an amount of about 30% to about 40% by volume (e.g., about 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, or any range or value therein); at least one alcohol in an amount of about 30% to about 40% by volume (e.g., about 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, or any range or value therein); and at least one emulsifier (i.e., total amount of all emulsifiers) in an amount of about 5% to about 15% by volume (e.g., about 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, or any range or value therein). In some embodiments, the total amount of hydrophobic base in the formulation may be in a range from about 25%-35% by volume of the formulation or about 30% to about 40% by volume of the formulation. In some embodiments, the total amount of alcohol in the formulation may be in a range from about 25% to about 40% by volume of the formulation or about 30% to about 40% by volume of the formulation. In some embodiments, the total amount of emulsifier in the formulation may be in a range from about 5% to about 15% by volume of the formulation.

[0029] 2-undecanone (also known as methyl nonyl ketone) comprises the structure of wherein R is C_9.



(I)

[0030] The formulations of the invention comprise a hydrophobic base. "Hydrophobic base" as used herein refers to a natural and/or synthetic fat, oil, and/or the like. Any suitable hydrophobic base may be used in a formulation of the present invention. In certain embodiments of the present invention, a formulation may comprise two or more hydrophobic bases, such as, but not limited to, 2, 3, 4, 5, or more hydrophobic bases. Exemplary hydrophobic bases include, but are not limited to, branched and unbranched hydrocarbons, hydrophobic polymers, fatty acids, fatty acid esters, monoglycerides, diglycerides, and/or triglycerides. A hydrophobic base may be partially or wholly saturated and/or unsaturated.

[0031] Additional exemplary hydrophobic bases may include, but are not limited to plant and/or vegetable oils, animal oils, and/or mineral oil. A plant-based oil may be any oil obtained from a plant or plant part (e.g., oil from seeds, flowers, fruit, leaves, bark, roots, etc). Exemplary plant oils include avocado oil, borage oil, canola (rapeseed) oil, castor oil, chamomile oil, coconut oil, corn oil, cottonseed oil, rapeseed oil, evening primrose oil, safflower oil, sunflower oil, soybean oil, sweet almond, palm oil, palm kernel oil, arctium lappa seed oil, sesame oil, borgo officialis seed oil, brassica campestris oleifera oil, brevoortia oil, bubulum oil, cistus ladaniferus oil, elaeis guineensis oil, almond oil, pine oil, olive oil, peanut oil, flax seed oil, wheat germ oil, hemp oil, jojoba oil, linseed oil, macadamia oil, neem oil, argan oil, grape seed oil, thistle oil, and/or algal oil and/or any combination thereof.

[0032] In some embodiments, the molecular weight of a hydrophobic base useful with this invention may range from about 100 g/mol to about 1000 g/mol. Thus, in some embodiments, the molecular weight of a hydrophobic base useful with this invention may range from about 100 g/mol to about 750 g/mol, about 150 g/mol to about 750 g/mol, about 150 g/mol to about 500, about 150 g/mol to about 300 g/mol, and any range or value therein.

[0033] In some embodiments, a hydrophobic base may be a 16 carbon or 18 carbon compound or a combination thereof. In some embodiments, a hydrophobic base may be about 10-20% saturated. In some embodiments, a hydrophobic base may be about 75-90% unsaturated. In some embodiments, a hydrophobic base may have a density of about 0.91 to 0.93 g/cm3 between the temperatures of 15° C. and 25° C.

[0034] In some embodiments, a formulation of the invention comprises at least one hydrophobic base that is a plant-based oil. In some embodiments, the at least one plant-based oil of a formulation of the invention may comprise, consist essentially of, or consist of soybean oil. In some embodiments, the at least one seed oil of a formulation of the invention may comprise, consist essentially of, or consist of cottonseed oil. In some embodiments, the at least one seed oil of a formulation of the invention may comprise, consist essentially of coconut oil. In some embodiments, the at least one seed oil of a formulation of the invention may comprise, consist essentially of, or consist of soybean oil and cottonseed oil. In some embodiments, the at least one seed oil of a formulation of the invention may comprise, consist essentially of, or consist of soybean oil and coconut oil. In some embodiments, the at least one seed oil of a formulation of the invention may comprise, consist essentially of, or consist of cottonseed oil and coconut oil. In some embodiments, the at least one seed oil of a formulation of the invention may comprise, consist essentially of, or consist of soybean oil, cottonseed oil and coconut oil.

[0035] A formulation of the invention comprises at least one alcohol. Non-limiting examples of alcohols useful with the invention include ethanol, isopropyl alcohol, t-butyl alcohol, propyl alcohol, benzyl alcohol or any combination thereof. In some embodiments, the alcohol may come from denatured alcohol and/or vodka. In some embodiments, the at least one alcohol of a formulation of the invention may comprise, consist essentially of, or consist of ethanol. In some embodiments, the at least one alcohol of a formulation of the invention may comprise, consist essentially of, or consist of denatured ethanol. In some embodiments, the at least one alcohol of a formulation of the invention may comprise, consist essentially of, or consist of isopropyl alcohol. In some embodiments, the at least one alcohol of a formulation of the invention may comprise, consist essentially of, or consist of t-butyl alcohol. In some embodiments, the at least one alcohol of a formulation of the invention may comprise, consist essentially of, or consist of propyl alcohol. In some embodiments, the at least one alcohol of a formulation of the invention may comprise, consist essentially of, or consist of benzyl alcohol. In some embodiments, the at least one alcohol of a formulation of the invention may comprise, consist essentially of, or consist of ethanol and isopropyl alcohol. In some embodiments, the at least one alcohol of a formulation of the invention may comprise, consist essentially of, or consist of ethanol, isopropyl alcohol and denatured alcohol.

[0036] Any denatured alcohol may be used in a formulation of the invention. In some embodiments, a "specially denatured alcohol" (SDA) may be used. In some embodiments, the denatured alcohol can be 95% ethanol and 5%

isopropanol by volume. In some embodiments, the denatured alcohol can be 90% ethanol and 10% methanol by volume.

[0037] In some embodiments, a formulation of the invention comprises an equal amount of alcohol to hydrophobic base. In some embodiments, a formulation of the invention comprises about 3%-10% more alcohol than hydrophobic base (e.g., 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10% vol./vol. more alcohol than hydrophobic base) (e.g., about 40% alcohol to about 30% hydrophobic base, about 40% alcohol to about 35% hydrophobic base, about 38% alcohol to about 30% hydrophobic base, about 35% alcohol to about 32% hydrophobic base, about 35% alcohol to about 30% hydrophobic base, about 35% alcohol to about 25% hydrophobic base, about 30% alcohol to about 25% hydrophobic base, about 34% alcohol to about 30% hydrophobic base and the like). In some embodiments, a formulation of the invention comprises about two times the amount of hydrophobic base to alcohol (e.g., about 30% hydrophobic base to about 15% alcohol, about 40% hydrophobic base to about 20% alcohol, about 50% hydrophobic base to about 25% alcohol; about 60% hydrophobic base to about 30% alcohol, and the like).

[0038] A formulation of the invention further comprises emulsifiers to assist in maintaining a stable monophasic system. Non-limiting examples of an emulsifier include polysorbate 20, sorbitan oleate, potassium sorbate, sodium benzoate, polysorbate 80, Tween 20, Tween 21, Tween 40, Tween 60, Tween 61, Tween 80, Tween 81, Tween 85; and/or sodium dodecyl sulfate (SDS). In some embodiments, at least one an emulsifier a formulation of the invention may comprise, consist essentially of, or consist of polysorbate 20, sorbitan oleate, potassium sorbate, and/or sodium benzoate. In some embodiments, the at least one emulsifier of the formulation may comprise, consist essentially of, or consist of polysorbate 20. In some embodiments, the at least one emulsifier of the formulation may comprise, consist essentially of, or consist of sorbitan oleate. In some embodiments, the at least one emulsifier of the formulation may comprise, consist essentially of, or consist of polysorbate 20 and sorbitan oleate.

[0039] In some embodiments, the formulations of the invention described herein may further comprise at least one of oil of lemon eucalyptus (OLE), p-methane-3,8-diol, and/or geraniol in an amount of about 5% to about 15% by volume (e.g., about 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, or any range or value therein; e.g., about 7% to about 12%, about 7% to about 13%, about 7% to about 14%, about 8% to about 11%, about 8% to about 12%, about 8% to about 13%, or any range or value therein). In some embodiments, the formulation may comprise geraniol in an amount of about 5% to about 15%. In some embodiments, the formulation may comprise OLE in an amount of about 5% to about 15%. In some embodiments, a formulation of the invention may comprise p-methane-3,8-diol in an amount of about 5% to about 15%. In some embodiments, a formulation of the invention may comprise geraniol and OLE in an amount of about 5% to about 15%. Thus, in exemplary embodiments, a formulation of the invention may comprise 2-undecanone in an amount of about 5% to about 50%, about 5% to about 30%, about 10% to about 20% or about 12% to about 18% by volume, or any range or value therein; at least one hydrophobic base in an amount of about 5% to about 65%, about 25% to about 40%, about 25% to about 35%, or about 27% to about 33% by volume, or any range or value therein; at least one alcohol in an amount of about 5% to about 50%, about 25% to about 40%, about 30% to about 40% or about 32% to about 38% by volume, or any range or value therein; at least one emulsifier in an amount of about 5% to about 15%, about 6% to about 13%, about 7% to about 12% or about 8% to about 12% by volume, or any range or value therein; and at least one of OLE, p-methane-3,8-diol, and/or geraniol in an amount of about 5% to about 15%, about 6% to about 13%, about 7% to about 12% or about 8% to about 12% by volume, or any range or value therein. In some embodiments, a formulation of the invention may comprise 2-undecanone in an amount of about 12% to about 18% by volume; at least one hydrophobic base in an amount of about 28% to about 33% by volume, at least one alcohol in an amount of about 30% to about 35% by volume; at least one emulsifier in an amount of about 8% to about 12% by volume; and at least one of OLE, p-methane-3,8-diol, and/or geraniol in an amount of about 8% to about 12% by volume. In some embodiments, the formulations of the invention described herein comprise OLE in an amount of about 5% to about 15%, about 8% to about 12% or about 8% to about 13%, about 9% to about 12% by volume, and any range or value therein.

[0040] In some embodiments, a formulation of the invention may be anhydrous. In some embodiments, a formulation of the invention may comprise water in an amount less than 0.25% vol/vol. In some embodiments, a formulation of the invention may comprise water in a range from about 0.25% vol./vol. to about 50% vol./vol.

[0041] The formulations of the invention may be used to repel animals including, but not limited to, insects and ticks.

[0042] Accordingly, in some embodiments, the present invention provides a method of repelling an insect or a tick, comprising applying to a subject a formulation of the invention as describe herein in an amount effective to repel an insect or a tick. In some embodiments, an effective amount of a formulation of the invention can comprise by volume about 5% to about 50%, about 5% to about 40%, about 5% to about 30%, about 5% to about 20%, about 5% to about 17.5%, about 5% to about 15%, about 10% to about 50%, about 10% to about 40%, about 10% to about 30%, about 10% to about 20%, about 10% to about 17.5%, about 12% to about 17%, 13% to about 17%, 14% to about 16%, about 15% to about 50%, about 15% to about 40%, about 15% to about 30%, about 15% to about 20%, about 15% to about 17.5%, about 15% to about 18%, about 20% to about 50%, about 20% to about 40%, about 20% to about 30%, about 30% to about 50%, about 30% to about 40%, about 30% to about 50%, about 5%, about 6%, about 7%, about 8%, about 9%, about 10%, about 10.5%, about 11%, about 11.5%, about 12%, about 12.5%, about 13%, about 13.5%, about 14%, about 14.5%, about 15%, about 15.5%, about 16%, about 16.5%, about 17%, about 17.5%, about 18%, about 18.5%, about 19%, about 19.5%, about 20%, about 20.5%, about 21%, about 22%, about 23%, about 24%, about 25%, about 26%, about 27%, about 28%, about 29%, about 30%, about 35%, about 40%, about 45%, or about 50% of 2-undecanone, and any range or value therein.

[0043] In some embodiments, application of a formulation of the invention may be repeated as needed. Thus, in some embodiments, the application of the formulation may be repeated every six hours, every 12 hours, every 24 hours, every other day, every three days, once a week, and the like, as needed.

[0044] In some embodiments, the formulations of the invention may be used to repel animals generally including, but not limited to, insects, ticks, spiders, hunting spiders, and/or scorpions. In some embodiments, the formulations of the invention may be used for repelling insects and ticks, wherein the insect may be, for example, a mosquito, sand fly, filth fly, chigger, flea, black fly, deer fly, horse fly, lice (e.g., body lice, head lice), roach, ant, fire ant, gnat, no-see um, and/or chigger, and the like.

[0045] The term "mosquito" as used herein refers to any type of mosquito (e.g., *Anopheles, Aedes,* and *Culex*), including but not limited to Tiger mosquitoes, *Aedes aboriginis, Aedes Aegypti, Aedes, albopictus, Aedes cantator, Aedes sierrensis, Aedes sollicitans, Aedes squamiger, Aedes sticticus, Aedes vexans, Anopheles quadrimaculatus, Culex pipiens,* and/or *Culex quinquefavciatus.*

[0046] The term "tick" as used herein refer to any type of tick, including but not limited to, deer ticks, the American dog tick (*Dermacentor variabilis*), *Ornithodoros parkeri, O. moubata,* and/or *Dermacentor andersoni.*

[0047] The term "roach" as used herein refers to any type of roach, including but not limited to the American cockroach (*Periplaneta americana*), German cockroach (*Blattella germanica*), oriental cockroach (*Blatta orientalis*), wood cockroach (*Parcoblatta pennsylvanica*), brownbanded cockroach (*Supella longipalpa*), and/or smokybrown cockroach (*Periplaneta fuliginosa*).

[0048] In some embodiments, a formulation of the present invention may repel at least about 90% (e.g., at least about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 100%, and any value or range therein) (e.g., percent repellency) of the insects and/or ticks up to about seven hours (e.g., about 1 min, 5 min, 10 min, 15 min, 20 min, 25 min, 30 min, 35 min, 40 min, 45 min, 50 min, 55 min, 1 hr, 1.1 hrs, 1.2 hrs, 1.3 hrs, 1.4 hrs, 1.5 hrs, 1.6 hrs, 1.7 hrs, 1.8 hrs, 1.9 hrs, 2 hrs, 2.1 hrs, 2.2 hrs, 2.3 hrs, 2.4 hrs, 2.5 hrs, 2.6 hrs, 2.7 hrs, 2.8 hrs, 2.9 hrs, 3 hrs, 3.1 hrs, 3.2 hrs, 3.3 hrs, 3.4 hrs, 3.5 hrs, 3.6 hrs, 3.7 hrs, 3.8 hrs, 3.9 hrs, 4 hrs, 4.1 hrs, 4.2 hrs, 4.3 hrs, 4.4 hrs, 4.5 hrs, 4.6 hrs, 4.7 hrs, 4.8 hrs, 4.9 hrs, 5 hrs, 5.1 hrs, 5.2 hrs, 5.3 hrs, 5.4 hrs, 5.5 hrs, 5.6 hrs, 5.7 hrs, 5.8 hrs, 5.9 hrs, 6 hrs, 6.5 hrs, or 7 hrs or any range or value therein) after application of the formulation to a subject. Thus, in some embodiments, a formulation of the invention may be therapeutically effective (e.g., repels about 90% to 100% of insects and ticks) for about 4 hrs to about 7 hrs or more after application to a subject. In some embodiments, a formulation of the present invention may repel at least about 90% to 100% of insects (e.g., mosquitoes) for up to about 4 hrs to about 6 hrs, about 4 hrs to about 5 hrs, about 5 hrs to about 6 hrs, or any range or value therein after application to a subject. In some embodiments, a formulation of the present invention may repel at least about 90% to 100% of ticks for up to about 5 hrs to about 7 hrs, about 5 hrs to about 8 hrs, about 6 hrs to about 7 hrs, about 6 hrs to about 8 hrs, or any range or value therein after application to a subject.

[0049] As is well understood, the repellency and duration of repellency for ticks versus insects such as mosquitoes can be different. This is due in part to the fact that odorant receptors on mosquitoes are on the antennae found on the head while those for ticks are on the tarsi (feet) on the front pair of legs. When repellency studies are conducted on mosquitoes, the mosquitoes are flying toward the human subject, detect the repellent that has evaporated from the skin of the human subject into the air some distance from the host (decreasing by 1 divided by distance squared from the repellent origin on the skin), and moving away from the repellent source (the skin). Because the mosquito is moving rapid through space in the direction of the host cues, the chemical repellency (amount of repellent in air and its repellent potency based on its unit of repellent activity per mole) must be high for repellency to occur. In the case of ticks, they are already on the host surface (skin or hair) or on host clothing and potentially moving toward a surface protected by the repellent. In this case, they are essentially walking on their olfactory surface, which comes into almost direct contact with the repellent treated surface. The repellent level in the air away from the host is not important but only that on or near the skin surface. The formulation developed in this invention was designed to provide the maximum duration of repellency for mosquitoes but also at same time provide maximum duration of repellency for ticks. Thus, in some embodiments, tick repellent duration may be longer because the tick detection and response to the repellent is at the repellent source on the skin surface and the duration of exposure to the repellent per unit time is higher since the tick is crawling at a much lower linear rate than mosquitoes that are flying toward the host. The formulation of the invention is designed to provide a longer tick duration than that for mosquitoes than current commercial repellents but at the same time provide a mosquito repellent duration competitive with current commercial mosquito repellents with comparative percentage levels of different active ingredients. Thus, in addition to insect and mosquito repellency, the present invention provides long lasting tick repellency that is advantageous in many locations where ticks may pose a greater human health risk than mosquitoes relative to the transmission of human diseases (for example, in the US and Europe).

[0050] In some embodiments, a formulation of the present invention may repel about 90% to about 100%, about 95% to about 98%, about 95% to about 99%, about 95% to about 100%, about 96% to about 98%, about 96% to about 99%, about 96% to about 100%, about 97% to about 98%, about 97% to about 99%, about 97% to about 100%, about 98% to about 99%, about 98 to about 100%, or about 99 to about 100% of the insects and/or ticks and any range or value therein for up to about seven hours after application of the formulation to a subject. In some embodiments, a formulation of the invention may repel about 95% to about 99% of the insects and ticks for up to about six hours after application of the formulation to a subject. In some embodiments, a formulation of the invention may repel about 95% to about 99% of the insects and ticks for up to about five hours after application of the formulation to a subject. In some embodiments, a formulation of the invention may repel about 98% to about 100% of insects and ticks for up to about four hours after application of the formulation to a subject. In some embodiments, a formulation of the invention may repel about 95 to about 99% of the insects and ticks for more than six hours (e.g., 6, 7, 8, 9, 10, 11, 12 hrs or more) after application of the formulation to a subject.

[0051] In some embodiments, a formulation of the invention may repel about 90% to about 100% (90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 100%, and any range or value therein) of insects and ticks for up to about 16 weeks (e.g., about 6 hours, 7 hours, 8 hours, 9 hours, 10 hours, 11 hour, 12 hours, 18 hours, 1 day, 2 days, 3 days, 4

days, 5 days, 6 days, 1 week, 2 weeks, 3 weeks, 4 weeks, 5 weeks, 6 weeks, 7 weeks, 8 weeks, 9 weeks, 10 weeks, 11 weeks, 12 weeks, 13 weeks, 14 weeks, 15 weeks, 16 weeks or more, or any range or value therein) when applied to a substrate. Thus, in some embodiments, a formulation of the invention applied to a substrate may repel about 90% to about 100%, about 95% to about 98%, about 95% to about 99%, about 95% to about 100%, about 96% to about 98%, about 96% to about 99%, about 96% to about 100%, about 97% to about 98%, about 97% to about 99%, about 97% to about 100%, about 98% to about 99%, about 98 to about 100%, or about 99 to about 100%, or any value or range therein, of insects and/or ticks for up to about 16 weeks and possibly longer after application of the formulation to the substrate.

[0052] In some embodiments, a determination of the percent of insects or ticks repelled by the formulation of the invention can be through the use of in an "arm in the cage" test or a "filter paper" test.

[0053] Filter paper test. For the filter paper test, a 1 ft×1 ft×1 ft Plexiglas with a stocking sleeve covering one end (the one end that does not contain a Plexiglass side) (e.g., a plastic cage with four sides and a bottom and a top and one side having a stocking sleeve instead of a plastic side). The cage is filled with a sufficient number of mosquitoes (females only) so that when two grade number 1 filter paper discs (a control and a treated disc) (3.5 inches (9 cm) in diameter) are hung on the walls of the cage (on opposite sides of the cage), at least about 10 mosquitoes land per disc when the mosquitoes in the cage are excited into flight by blowing into the cage and then left undisturbed for 1 min. The assay is conducted at 25-27° C. under low light conditions (humidity is not regulated but typical lab conditions). The environmental conditions are the same throughout the studies.

[0054] For each study, one filter paper disc is treated with the repellent (500 microliters per disc) and the other with the same solvent (500 microliters per disc) used to deliver the repellent. To evenly distribute the test compound and control solvent, the filter paper in each case is treated by application of small droplets added evenly over the entire surface of the paper. Filter paper is particularly useful because it allows the droplets to spread out from the point of application by absorption and diffusion within the cellulose disc. Once the filter paper discs are treated, they are laid horizontal on a glass surface on the lab bench at room temperature of about 25° C. plus/minus 2° C. and 60% relative humidity.

[0055] At different times after treatment of the paper, the discs are hung in the cage (the control (treated with carrier solvent only) is hung first and the disc carrying the formulations of the invention are hung second. The researcher handling the discs wears powder-free nitrile examination gloves. The process of placing the discs in the cage and also blowing into the cage by the researcher causes the mosquitoes to fly. Once the researcher removes his/her hands, the number of mosquitoes landing and remaining on the disc for more than three seconds is considered not to be repelled and is counted as such. The test is run over a fixed time, e.g., about 1 min). The researcher blows into the cage one additional time at the 30 sec interval into the 1 min observation period. The discs are removed in the same order that they were placed into the test cage by the researcher who is wearing a new pair of clean gloves. Since the discs are present in the cage for no more than about 1 min, any repellent released into the air will have minimum effects on the physiology of the mosquito. Percentage repellency is calculated as the number of mosquitoes landing on the control paper minus the number of mosquitoes landing on the treatment divided by the number of mosquitoes on the control paper times 100. Since the assay takes only 1 min to conduct, multiple compounds can be tested on the same cohort of mosquitoes. Periodically, the assay is checked to determine that no positional bias is occurring in the cage (e.g., the mosquitoes may favor one filter paper versus another due to the position of one of the filter paper discs relative to a light source). Therefore, control experiments are conducted from time to time to determine if both filter paper discs untreated attract approximately the same number of mosquitoes.

[0056] Tick repellency filter paper test. Tick repellency was also determined using a filter paper test. In this case, naïve, unfed, host-seeking (as determined by raised forelegs in response to the investigator's breath), mixed sex, adult ticks were used in all trials. Adult ticks (all three species) for use in repellency studies were held in plastic vials at about 28° C., about 75% relative humidity (RH), and a photoperiod of 14 hr light: 10 h dark, including dusk and dawn periods (60 min each). Behavioral bioassays were conducted at 25_C, 65% RH, and in complete darkness (except during the approximately 5 seconds to monitor tick distribution).

[0057] Ticks were allowed to choose between a treated and untreated filter paper surface. Tests were conducted in 63.6 cm^2 Petri plate lids lined with two 31.8 cm^2 semi-circle pieces of filter paper (Whatman no. 1), each treated with the formulations of the invention and allowed to dry for 3 h at room temperature under a fume hood. At 3 h, six ticks were placed in each arena along the line formed by the junction of treated and untreated papers. Distribution of ticks was recorded every 5 min from 5 to 30 min after the introduction of ticks to test arenas. An untreated control was used to determine the distribution of ticks in the absence of a repellent. Trials were repeated six times.

[0058] Arm-in-the cage-test. For the arm in the cage test, the arm of a human subject (treated and untreated) is retained in the cage for 1 min.

[0059] A fixed amount of a test compound (e.g., formulation of the invention) (1 ml) is applied to one forearm by a researcher (the observer) who is wearing non-allergenic gloves on both hands. The observer applies the test compound using a pipette with a sterile tip and then spreads and massages the test compound into the skin from the elbow to the wrist on one arm of the subject (i.e., test arm).

[0060] At different times after application of the formulations of the invention, the subject applies a non-allergic glove to the hand on the test arm and inserts their arm into a cage of up to 200 female mosquitoes for about 1 min to about 5 min (e.g., about 1, 2, 3, 4, 5 min). A glove is used to cover the hand because the hand is not part of the assay—only the arm is used in the assay. The length of time may be varied depending on the activity of the mosquito or the mosquito species. It is known that different mosquito species and exact age of mosquito as well as unknown environmental and genetic variation in mosquitoes can affect activity (flying, landing and trying to bite). The repellency may be measured at multiple times post-treatment. Thus, the test may be run, for example, at about 4 hr, 5 hr, 6 hr, 7 hr, 8 hr, 9 hr, 10 hr, 11 hr, or 2 hr post-treatment. Between tests,

the subject removes and disposes of the glove on the hand of the treated arm. For each time point, a new glove is used.

[0061] Mosquitoes that land on the skin for more than 2 seconds are counted as landing and the subject will move their arm in the cage so that the mosquito is not provided the opportunity to bite. An observer will count the number of landings.

[0062] The measurement of landings on the untreated arm (control arm) will precede the measurements made on treated arm. The subject will also have a glove on their untreated arm at each testing point, which will be replaced with a new glove for each time point.

[0063] The experiment is designed to observe about 200 or more landings on the untreated arm within one min. The mosquitoes are not allowed to take a blood meal and are prevented from doing so by shaking the arm. Any time a mosquito touches the arm, it is counted as a landing; that is, the mosquitoes do not have to land on the skin with their feet. The percentage repelled is determined by subtracting the number of insects, for example, mosquitos, that land on the treated arm from the number of mosquitos that land on the untreated arm and dividing this sum by the number of mosquitos that land on the untreated arm times 100.

[0064] The mosquitoes used in all tests are lab reared, disease free, female, and have not fed on blood of any type before the tests are conducted.

[0065] Thus, in some embodiments, a formulation of the present invention repels about 90% to about 100%, about 95% to about 98%, about 95% to about 99%, about 95% to about 100%, about 96% to about 98%, about 96% to about 99%, about 96% to about 100%, about 97% to about 98%, about 97% to about 99%, about 97% to about 100%, about 98% to about 99%, about 98 to about 100%, or about 99 to about 100% of the insects and/or ticks about four hours to about 7 hours after application of the formulation to an arm in the arm in the cage test, and any range or value therein. In some embodiments, a formulation of the invention repels about 100% of the insects and ticks about four hours after application of the formulation to an arm in an arm in the cage test. In some embodiments, a formulation of the invention repels about 99% of the insects and ticks about five hours after application of the formulation to an arm in the arm in the cage test.

[0066] Subjects to which the formulations of the present invention may be applied include human subjects and other animals (e.g., dogs, cats, horses, cattle). In some embodiments, a formulation is applied to a subject's skin and/or hair. In some embodiments, when the formulation of the invention is applied to a subject's hair the percent and time period of repellency may be greater than for application of the formulation to the subject's skin.

[0067] The formulations of the invention may be applied to subjects directly or indirectly, such as by applying the formulation to the skin or hair of the subject, or by applying the formulation to an article worn by or otherwise protecting/shielding the subject. As an example, a formulation of the invention may be applied to netting or screening that protects a subject.

[0068] A formulation of the invention may be applied to any substrate for which repelling animals, including insects and ticks is desired.

[0069] In some embodiments, a substrate may include, but is not limited to, fabrics (e.g., clothing, towels, linens, bed clothing, draperies, curtains, and the like); cotton products, wrist bands, wood products, furniture (e.g., chairs, mattresses, box springs, etc.), carpets, floors, soil and/or plants, containers, walls, base boards, air, soil, neting, paper, trash containers, dumps, tents, paint, etc.

[0070] Application to subjects or substrates may be carried out by any means that transfers the formulation to the subject or substrate, including but not limited to, spraying, misting, spreading, dusting, pouring, dipping, sprinkling, rubbing, fumigating, rolling, atomizing, and the like.

[0071] The formulations of the invention may be formulated in a variety of ways, including but not limited to liquids, powders, solids, emulsions, mousses, granules, dusts, a suspension, an aerosol (e.g., powdered, liquid), a shampoo, a conditioner, a creme rinse, a mousse, a foam, a lotion, a gel, a powder (e.g., a dry shampoo), a shampoo, a conditioner, a creme rinse, a paste, or an ointment. The formulations of the invention may be incorporated into or coated onto textiles, plastics, latex, rubber, cellulose, wicks, candles, paint, heat and air dispensers.

[0072] The invention will now be described with reference to the following examples. It should be appreciated that these examples are not intended to limit the scope of the claims to the invention, but are rather intended to be exemplary of certain embodiments. Any variations in the exemplified methods that occur to the skilled artisan are intended to fall within the scope of the invention.

EXAMPLES

Example 1

[0073] Procedures for testing repellent activity against the yellow fever mosquito (*Aedes aegypti*), the Asian tiger mosquito (*Aedes albopictus*), and *Anopheles gambiae* (a malaria vector).

[0074] A fixed amount of repellent (1 ml) is applied to one forearm of the subject by a researcher (the observer) wearing non-allergenic gloves on both hands. The observer will apply the repellent using a Gilson pipetter with a sterile tip and then spread and massage the repellent into the skin using the pipette tip as a spreading device spreading the repellent from the elbow to the wrist on one arm of the subject (the test arm).

[0075] At different times after application of the repellent up to 12 h, the subject will apply a non-allergic glove to the hand on the test arm and then insert their arm into a cage of 200 female mosquitoes for 1-5 min. The length of time might be different depending on the activity of the mosquito or species; mosquito species and exact age of mosquito as well as unknown environmental and genetic variation in mosquitoes can affect activity (flying, landing and trying to bite). Post treatment measurements are made at 4, 5, 6, 7 and 8 h and possibly 12 h if repellency remains greater than 70% at 8 h. Between tests, the subjects remove and dispose of their glove on the hand of the treated arm. A new glove is used for each time point of testing.

[0076] Mosquitoes that land on the skin for more than 1 sec. will be considered ready to bite and the subject will move their arm in the cage so the mosquito leaves the skin to avoid actual biting of the subject. An observer will count the number of landings.

[0077] The subject has a glove on their untreated or control arm at each testing point. The insertion of the untreated arm will precede the treated arm and number of landings recorded as described. The glove is removed from the untreated arm immediately after removal from the cage as also will be the case for the treated arm. A new glove is used for each time point.

[0078] The study is stopped when percentage repellency is less than 50%. Percentage repellency is calculated based on the number of mosquitoes landing on the untreated arm minus the number landing on the treated arm divided by the number landing on the untreated arm times 100.

[0079] The mosquitoes used in the study are lab reared, disease free, adult female, and have not fed on blood of any type before the tests are conducted. Since the mosquitoes are not allowed to bite the subjects, there is also no chance for reactions to mosquito bites.

[0080] The mosquitoes are killed by freezing after the testing, autoclaved and disposed in the regular trash.

TABLE 1

Results of repellency tests for selected formulations

		Insect *Aedes Aegypti*					
Formula Name	Active ingredients	1 hr	2 hr	3 hr	4 hr	6 hr	8 hr
Soybean 1	8% undecanone, 5% geraniol, 35% soybean oil, 52% isopropyl alcohol				*88.5% repellent		
Soybean 2	8% undecanone, 5% geraniol, 50% soybean oil, 37% isopropyl alcohol				*94.5% repellent		
Soybean 3	8% undecanone, 5% geraniol, 65% soybean oil, 22% isopropyl alcohol					*100% repellent	
Soybean 4	8% undecanone, 5% geraniol, 87% soybean oil					*100% repellent	

*200 mosquitoes in the cage, calculation of percentage repellency based on number of landings on treated arm versus untreated arm (see example 1 for calculation details); undecanone = 2-undecanone

Example 2

[0081] A combination of 2-undecanone with soybean oil and geraniol in the ranges tested provided an increased repellency over 2-undecanone in cotton seed soil and over 2-undecanone in soybean oil using polar solvents like water. In Table 1, the four component formulations were problematic for the following reasons: (i) the product separated into multiple phases when allowed to stand at room temperature; (ii) the product needed to be shaken to reform an emulsion before use (to re-establish a single phase) and as a result of (ii), the product (a) was predicted to have delivery issues as a commercial repellent possibly causing harm to users from the accidental application of neat ingredients and (b) would require specialized development of a new delivery system to apply the repellent because of the chemical degradation of the container parts by the separated 2-undecanone and/or geraniol (which adds to the cost of product development for the delivery system, potentially would result in development challenges that might not be solvable (including issues with having to shake the bottle before use) which is not consistent with industry standards for these type of products); further, the product (iii) was too viscous to be applied by aerosol; (iv) had a poor skin feel—felt oily and was uncomfortable to human test subjects; (v) had a high rate of contact transfer from skin to other materials like clothing that might affect product efficacy for mosquito and tick protection; (vi) did not achieve adequate mosquito repellency 100% at 5 h post treatment; (vii) had a strong, pungent smell which would not be desirable to users and hard to mask with other scents to make a final product with an acceptable odor; and (viii) produced undesirable skin reactions (all in part or whole from list given). Thus, the formulations in Table 1 demonstrated that alcohol could be used to decrease soybean oil content and therefore, improve feel on skin. However, these formulations achieved a repellency similar to that of the plant-based oil only.

Example 3

[0082] Addition of alcohol breaks the heavy oily feel of the formulation and reduces the ease of transfer of the repellent from treated skin to items like clothing that might come into contact with treated skin. While, Table 2 included a formulation with a different alcohol to plant-based oil concentration than those in Table 1, the negative aspects of the four component system discussed above in Example 2 were still present, including the separation of the formulation into different phases when left standing. Further, a higher level of plant-based oil was still needed to achieve the desired repellency; however, this higher amount of oil creates the undesirable greasy/oily feel discussed above. Thus, the combined data from Tables 1-2, helped establish the relationship between alcohol level and repellency in a

dose dependent manner and assisted in moving closer to an alcohol/oil ratio that would provide 100% repellency and the best feel on the skin.

TABLE 2

Results of repellency tests for selected formulations

	Insect *Aedes aegypti*					
Formula Name	Active, ingredients	1 hr	2 hr	3 hr	4 hr	6 hr	8 hr
Soybean 5	8% undecanone, 5% geraniol, 25% soybean oil, 62% isopropyl alcohol				*73.5% repellent		
Soybean 1	8% undecanone, 5% geraniol, 35% soybean oil, 52% isopropyl alcohol				*88.5% repellent		
Soybean 2	8% undecanone, 5% geraniol, 50% soybean oil, 37% isopropyl alcohol				*94.5% repellent		
Soybean 3	8% undecanone, 5% geraniol, 65% soybean oil, 22% isopropyl alcohol					*100% repellent	
Soybean 4	8% undecanone, 5% geraniol, 87% soybean oil					*100% repellent	

*200 mosquitoes in the cage, calculation of percentage repellency based on number of landings on treated arm versus untreated arm (see example 1 for calculation details); undecanone = 2-undecanone.

Example 4

[0083] The addition of specific emulsifiers prevented separation into multiple phases and improved the feel on the skin and the reduced transfer rates were maintained. However, the repellency of the formulations was reduced (Table 3). Also, the other negative characteristics (see Example 2, above) were still present. These data demonstrate the use of commercial grade denatured alcohol (ethanol) instead of isopropyl alcohol in the formulations. Based on dose response data in Table 1 and Table 2, it is believed that the reduced repellency of the formulation was a result of reducing the percentage soybean oil.

TABLE 3

Results of repellency tests for selected formulations

Insect	*Aedes aegypti*			
Formula Name	Active, ingredients	Control	4.0 hr	% repellency
Soybean 6	15% 2-undecanone, 5% polysorbate 20, 20% soybean oil, 55% denatured alcohol, 5% geraniol	205 landing/ min	29 landing/ min	*86%

TABLE 3-continued

Results of repellency tests for selected formulations

Insect	*Aedes aegypti*			
Formula Name	Active, ingredients	Control	4.0 hr	% repellency
Soybean 6	15% 2-undecanone, 5% polysorbate 20, 20% soybean oil, 55% denatured alcohol, 5% geraniol	240 landing/ min	139 landing/ min	*42%

*200 mosquitoes in the cage, calculation of percentage repellency based on number of landings on treated arm versus untreated arm (see example 1 for calculation details).

Example 5

[0084] Further experiments adjusting oil to alcohol ratio was needed to improve the desired repellency, maintain positive skin feel, to minimize accidental transfer from treated skin, to allow a stable monophasic system, and to eliminate issues associated with delivery of the repellent (issues as discussed in Example 2, above), see Table 4.

TABLE 4

Results of repellency tests for selected formulations

Insect	*Aedes aegypti*			
Formula Name	Active, ingredients	Control	4.0 hr	% repellency
Soybean 7	15% 2-undecanone, 5% polysorbate 20, 30% soybean oil, 40% denatured alcohol, 5% geraniol, 5% sorbitan oleate	372 landing/ min	23 landing/ min	94%
Soybean 7	15% 2-undecanone, 5% polysorbate 20, 30% soybean oil, 40% denatured alcohol, 5% geraniol, 5% sorbitan oleate	292 landing/ min	45 landing/ min	85%

*200 mosquitoes in the cage, calculation of percentage repellency based on number of landings on treated arm versus untreated arm (see example 1 for calculation details).

Example 6

[0085] By further reducing the percentage of alcohol and adjusting other components, a desired repellency of over 95% was achieved in combination with a stable monophasic system, which addressed each of the issues raised in Example 2 (above).

[0086] The formulations of the invention are advantageous in that they do not easily rub off the skin and do not result in irritation to the skin when applied. Additionally, the formulations of this invention have an excellent feel and better activity than other known insect repellent formulations (e.g., BioUD, as shown in "arm in cage" studies). Thus, for example, as reported in Witting-Bissinger et al. (J. Med. Entomol., 45:891-898 (2008)) a commercial composition of 7.75% 2-undecanone (BioUD) had a mosquito repellency of about 70% at 5 hours post application as compared to 99% at 5 hours for the formulations of the present invention. Notably, in Witting-Bissinger et al., the cages contained 50 mosquitoes as compared to 200 in the cages used to test the formulations of the present invention. Thus, the formulations provided better repellency under greater biting pressure.

Example 7

[0087] Additional Testing of the Formulations of the Invention

[0088] In this example, a formulation comprising 15% 2-undecanone, soybean oil, polysorbate 20, sorbitan oleate, oil of lemon eucalyptus and denatured alcohol was tested.

[0089] A colony of *Aedes aegypti* (L.) mosquitoes used in the bioassay was established from field-collected eggs from New Orleans, La., in 2003. At 6 to 8 month intervals, adults reared from field-collected eggs were added to the mosquito colony to sustain genetic diversity. Larvae were reared as described by Trexler et al. (2003). Mosquito colonies were maintained in the Dearstyne Entomology laboratory at 28° C., 75% relative humidity, and a photoperiod of 14:10 (L:D), including two twilight periods (60 min each). Adult females used in the experiments were provided 10% sucrose solution ad libitum and were never allowed to blood feed.

[0090] The testing procedures were as follows. The test area was the surface of the arm from the elbow to the wrist (hands were covered with non-allergenic gloves). Test areas were washed with soap and water then swabbed with 70% ethanol before application of each repellent. The ethanol was allowed to evaporate completely before repellent treatments. Control arms were cleansed in the same manner. For all studies on human skin, repellents were applied at a rate of 1 ml/600 cm2. For each test, mated nulliparous host-seeking (non-blood fed) female mosquitoes aged between 6 and 18 d were released into a square 27,000 cm^3 Plexiglas cage with a sleeved opening on one side for insertion of a subject's arm. The cage contained only the test mosquitoes during the testing period (no nectar or water source).

[0091] For the arm in cage test, the arm of the human subject (treated versus untreated control) was inserted into the cage for 1 minute. Tests were conducted at 25° C., 60-70% relative humidity and under florescence laboratory lighting. A fixed amount of the test compound (1 ml) was applied to one forearm by a researcher (the observer) who was wearing non-allergenic gloves on both hands. The observer applied the test compound using a P1000 Pipetteman with a sterile tip and then spread (and massaged into the skin using the pipette tip) from the elbow to the wrist and one arm of the subject (i.e., the treatment arm). Prior to the study, the pipette calibration was validated by transferring distilled water to an analytical balance (+0.0001 grams) and was within the standard accuracy and precision of the manufacturer (<2% error). Nothing was applied to the other arm of the subject which was the control arm.

[0092] At different times after application of the formulation, the subject applied a non-allergic glove to the hand on the control and test arm and just afterward, inserted the arm into a cage (control arm first and then immediately the treatment arm); the cage contained up to 200 female mosquitoes. Each arm was inserted for 1 minute. The repellency was measured at multiple times post-treatment. Bioassays were conducted for each subject on the same cage of mosquitoes; different cages for different subjects. The time between bioassays was no shorter than 30 minutes and typically at least an hour. Insertion of the control versus treated arm did not cause a reduction in landing counts on the control arm at the different times tested. Between tests, the subject removed and disposed of the glove. For each time point, a new glove was used. The purpose of the glove was to prevent mosquito biting the hand, and no mosquito blood feeding on the hand was reported for any bioassay. The subject with a treated arm was encouraged to sit quietly and minimize physical activity.

[0093] Mosquitoes that touch the skin only are counted as landing; the mosquitoes do not have to stand on the skin with their legs to be considered a landing. When a landing was noticed where the mosquito is standing on the skin, the subject moved their arm in the cage in a rapid back and forth motion to prevent mosquito biting and blood feeding. No biting or blood feeding was noted in any of the bioassays. The observer counted the number of landings using a hand held numerical counter. The observer was positioned on the opposite side of the cage from the subject to best observe landings. The measurement of landings on the untreated arm (the control arm) preceded the measurements made on the treated arm. The mosquito density was adjusted to achieve 200 or more landings on the untreated arm within one minute.

[0094] Percentage repellency provided was calculated as follows: (control landings treatment landings/control landings)×100.

[0095] The formulation tested in each of Tables 5-8, below, comprised 15% undecanone, soybean oil, polysorbate 20, sorbitan oleate, oil of lemon eucalyptus and denatured alcohol.

TABLE 5

		At 4 hours		At 5 hours	
Subject	Control (land*/in)	Treatment (land/min)	% Repellency	Treatment (land/min)	% Repellency
1	320	19	94		
2	256	16	94		

*land = landings

TABLE 6

		At 4 hours		At 5 hours	
Subject	Control (land*/in)	Treatment (land/min)	% Repellency	Treatment (land/min)	% Repellency
1	312	1	99.7		
2	296	0	100		

*land = landings

TABLE 7

		At 4 hours		At 5 hours	
Subject	Control (land*/in)	Treatment (land/min)	% Repellency	Treatment (land/min)	% Repellency
1	156	2	98		
2	204	5	98		

*land = landings

TABLE 8

		At 4 hours		At 5 hours	
Subject	Control (land*/in)	Treatment (land/min)	% Repellency	Treatment (land/min)	% Repellency
1	216	3	99	2	99
2	468	3	99	4	99

*land = landings

The formulation of the invention was 99% effective for at least 5 hours in preventing mosquitoes even touching the subject's arm. In other trials with the formulation, the average repellency at 4 h was 95.3% (typical range 94 to 100%; Tables 5 to 7).

Example 8

[0096] The effect of the new formulations on the foraging behavior of ticks and mosquitoes.

[0097] In this example, a formulation comprising 2-undecanone, soybean oil, polysorbate 20, sorbitan oleate, oil of lemon eucalyptus and denatured alcohol was tested.

[0098] The objective of these laboratory studies was to quantify foraging behavior of mosquitoes and ticks in response to human study participants that were treated with the formulations of the invention.

[0099] In one study, mosquito landings were recorded on the untreated versus repellent-treated arms of human subjects. Exposures to mosquitos were made at 30-minute intervals, with each exposure lasting for 1 minute for treated arms and for 20 seconds for untreated control arms (to limit the number of bites received). Control exposures served to verify biting avidity of mosquitoes during the study. Complete Protection Time was defined as the time from application of the test material to a subject's skin until the exposure in which the first landing with intent to bite occurred that was followed by another landing with intent to bite within the same or the next exposure interval.

[0100] In a second study, crossings of individual ticks on untreated and repellent-treated arms of human subjects were recorded. Fresh ticks were first selected for avidity by initially checking that the ticks would traverse a specified distance on each subject's untreated arm within a three-minute period. "Qualifying" ticks were those that walked upward on an untreated arm, toward the elbow. Any tick that qualified was then moved to that subject's treated arm in order to challenge the repellent. Complete Protection Time was defined as the time from application until the exposure in which there was a crossing confirmed by another crossing within 30 minutes (First Confirmed Crossing). The earlier of these crossings was considered the end of Complete Protection.

[0101] Each study included four subjects. In the case of the study of the foraging behavior of mosquitoes, the negative control was the untreated arm of one subject. In the case of the study of the foraging behavior of ticks, the negative control was the untreated arm of each of the four subjects.

[0102] To minimize the chances of any potential substances (e.g., cosmetic products) on subject skin interfering with the test, each subject began by washing both arms with fragrance-free soap, rinsing with water, then washing both arms again with 70% ethyl alcohol in water, and finally rinsing with water. Arms were dried with plain paper towels prior to repellent applications. The topical repellent formulation of the invention was applied at a dosing rate of approximately of 1 mL per 600 cm2 of skin surface area. Accordingly, the total amount of material applied to each subject was determined by the surface area of the forearms of each subject.

Mosquito Testing.

[0103] Mosquito testing was against adult female *Aedes aegypti* from laboratory-reared disease-free populations.

[0104] The formulation tested in this example comprised 2-undecanone, soybean oil, polysorbate 20, sorbitan oleate, oil of lemon eucalyptus and denatured alcohol (e.g., "the test material"). The test material was applied from the wrist to the elbow of each participant using a 1 mL syringe. Specifically, the repellent was distributed onto the skin in drops and lines, and then spread with two fingertips of one hand until complete and even coverage was observed. For each application, fresh gloves were used.

[0105] Approximately 100 laboratory-reared adult female mosquitoes were used to challenge the repellent in each exposure interval. Prior to testing, mosquitoes were supplied with feeding bottles of 10% sucrose in water. Feeding bottles were removed 12 hours prior to testing in order to increase their foraging avidity. Mosquitoes were held in 12-inch (~30

cm) cubic screened cages. During each exposure period, repellents were challenged in sequence in a single cage of mosquitoes. At 30-minute intervals beginning shortly after repellents were applied, each participant began exposures by inserting a forearm into a cage. For treated arms, exposures were for one minute, and for untreated control arms the exposure was for 20 seconds. Two observers counted mosquito landings during each exposure. Control exposures served to verify avidity in mosquitoes. A 20 second exposure of untreated arms to the mosquitoes was estimated to represent about 50% of the number that would be expected to land in a full minute exposure.

[0106] Repellent failure was scored independently for each subject, and defined as the time after application at which the first bite occurred that was followed by another bite within that or either of the next two intervals (time of First Confirmed Bite (FCB)).

Tick Testing.

[0107] Tick tests used nymphal Deer Ticks, *Ixodes scapularis* from laboratory reared, disease-free populations and the formulation tested comprised 2-undecanone, soybean oil, polysorbate 20, sorbitan oleate, oil of lemon eucalyptus and denatured alcohol.

[0108] A single nymphal Deer Tick was used to challenge the repellent formulation of the invention during each exposure interval. Ticks were held in glass vials surrounded by water to contain them during the test, and fresh ticks were used for each challenge with a formulation of the invention. The formulation was challenged simultaneously in all subjects in 3 minute exposures commencing every 30 minutes beginning from the application of the test material.

[0109] Subjects manipulated ticks with small artist's paintbrushes, first placing them on an untreated arm to insure avid questing behavior in the absence of repellent, then placing them on the treated arm. In this way each subject served as their own negative control. Only avid ('qualified') ticks were used to challenge repellent formulations of the invention.

[0110] Crossings were measured by placing three dots on each subject's arms, 3-cm distant from one another, in a straight line (see, FIG. **1**). For a treated arm, repellent was present above the middle dot, and failure was defined as crossing 3-cm into the treated area, past the third dot. An exposure interval where a tick did continue past this third dot was scored as a crossing, and repellent was considered no longer effective at that point if that crossing was confirmed by another within that or either of the next two intervals (First Confirmed Crossing, or FCC). For controls, the same crossing criterion was used to insure tick avidity, but no treatment (i.e., no repellent was present).

[0111] Repellent failure was scored independently for each subject, and defined as the time after application in which the first crossing occurred that was followed by another crossing in the next two intervals (First Confirmed Crossing).

Results

Mosquitoes

[0112] Of the four test participants, two received confirming landing with intent to bite, for Complete Protection Time (CPT) of 3 and 5 hours, respectively. The remaining two subjects withdrew voluntarily. One of the subjects that withdrew voluntarily received a single landing with intent to bite at 2 hours and withdrew voluntarily at 4.5 hours and the other received a single landing with intent to bite at 6 hours and then withdrew. With two subjects experiencing repellent failures and two withdrawing before failure, only a conservative mean CPT value can be calculated. Mean±sd CPT was approximately 4.63±1.25 hours.

[0113] While exposures were initiated at 30-minute intervals, they were generally 5-10 min past the exact ½ hour intervals post application. Hence each of the hour values below understates protection duration by about 5-10 minutes. Accounting for minor exposure delays in each period increases the conservative mean of approximately 4.75 hours. Accepting that the subjects who withdrew could not have received confirming landing with intent to bite until at least 30 minutes after their withdrawal times, the minimum reasonable mean CPT calculation is probably about 5.0 hours.

Ticks

[0114] None of the four subjects experienced any crossings on the treated arms. All subjects withdrew after several hours of testing confirmed to evident prolonged complete protection. Two subject withdrew after 6.5 hours, and the other two withdrew after 7 hours. The mean±sd CPT against ticks was 6.75±0.25.

[0115] Against ticks, no subjects tested their treatment all the way to failure. Thus, longer protection times could have been recorded had all subjects done so. Nonetheless, the average protection time among the data gathered was reasonably long and showed no signs of failure.

[0116] In conclusion, the topical repellent formulation of the invention was effective against *Aedes aegypti* mosquitoes for about 5 hours, and against *Ixodes scapularis* ticks for at least a few additional hours more than observed for the mosquitoes. We regard this duration of repellency against ticks to be strong.

Example 9

[0117] Animal Toxicity Tests

[0118] Any formulation for use as a repellant in addition to providing repellency at the same time has to be compatible to use on humans without adverse effects including not only skin but clothing, hair and accidental application to eyes and other sensitive surfaces on the body. It must also have no toxic effects and also have an acceptable relative to smell and feel on the skin and also relative to retention on treated surfaces with resistance to removal from typical rubbing that might occur to skin on different parts of the body. The formulations of the present invention were tested to show that they meet all of these criteria.

Test I. Skin Sensitization Study in Guinea Pigs

[0119] A formulation of the invention comprising 2-undecanone, soybean oil, polysorbate 20, sorbitan oleate, oil of lemon eucalyptus and denatured alcohol was tested for dermal sensitization potential utilizing a Buehler Technique Guinea Pig Sensitization Protocol. The test substance was evaluated for sensitization potential by applying 0.4 ml at a 100% concentration directly into Hilltop Chambers® and applying them to the clipped left shoulder of twenty albino guinea pigs in the following manner: The animals were held

gently, and the chambers were applied as quickly as possible to the clipped left shoulder. The chambers were secured with Micropore tape and further secured with Kendall adhesive tape. Approximately six hours later, the tape and chambers were removed. Two additional induction doses were conducted following the same procedure, at weekly intervals.

[0120] Two weeks after the final application the animals received a topical primary challenge dose (6 hour contact) of the formulation at 100% concentration, on a naive site located on the right shoulder. Animals were scored for irritation at 24 and 48 hours after initiation of the primary challenge application.

[0121] Ten guinea pigs served as a naive control group, and remained untreated through the induction phase. Six naive control animals received only the primary challenge dose, at a 100% concentration. The four remaining guinea pigs were designated for a re-challenge, if necessary.

[0122] Following primary challenge with the formulation at 100% concentration, the incidence of grade 1 response or greater in the test group (0 of 20) was compared to that of the naive control group (0 of 6). The incidence and severity of these responses were not significantly greater than those produced by the naive control group indicating that sensitization had not been induced.

Test II. Acute Inhalation Toxicity Study in Rats

[0123] A formulation of the invention comprising 2-undecanone, soybean oil, polysorbate 20, sorbitan oleate, oil of lemon eucalyptus and denatured alcohol was tested for acute inhalation toxicity in accordance with OPPTS Guideline 870.1300 and OECD Guideline 403 at a targeted level of 2.0 mg/L of air by exposing five male and five female rats for a four hour period. The actual gravimetric concentration of the study was 2.33 mg/L of air. No mortality was observed during the 14 day observation period. The inhalation LC50 of the formulation was found to be greater than 2.33 mg/L of air for a 4 hour period. In accordance with the OPPTS Guidelines, formulation of the invention would be classified as EPA Toxicity Category IV for acute inhalation toxicity.

Test III. Acute Dermal Toxicity Study in Rabbits

[0124] A formulation of the invention comprising 2-undecanone, soybean oil, polysorbate 20, sorbitan oleate, oil of lemon eucalyptus and denatured alcohol was tested for acute dermal toxicity according to OPPTS, OECD Guidelines. The formulation, an amber liquid, was administered by dermal application at a dose of 5.0 g/kg body weight to five male and five female rabbits. No mortality occurred during the 14 day observation period. The acute dermal LD50 was found to be greater than 5.0 g/kg body weight. The formulation of the invention would be classified as Toxicity Category IV for dermal toxicity

Test IV. Acute Oral Toxicity Study in Rats

[0125] A formulation of the invention comprising 2-undecanone, soybean oil, polysorbate 20, sorbitan oleate, oil of lemon eucalyptus and denatured alcohol was tested for acute oral toxicity in accordance with OPPTS 870.1100 and OECD 425 Guidelines. The formulation, an amber liquid, was administered by oral gavage at a 5000 mg/kg body weight limit dose level, according to the Agency's Up and Down Procedure to female rats. The acute oral LD50 was determined to be greater than 5000 mg/kg body weight. Therefore, in accordance with OPPTS Guidelines, the formulation of the invention meets the requirements for EPA Toxicity Category IV for acute oral toxicity.

Test V. Acute Dermal Irritation Study in Rabbits

[0126] A formulation of the invention comprising 2-undecanone, soybean oil, polysorbate 20, sorbitan oleate, oil of lemon eucalyptus and denatured alcohol was tested for primary dermal irritation in accordance with OPPTS and OECD Guidelines. The formulation, an amber liquid, was applied at a dose of 0.5 ml to an unabraded site on a clipped area of each of three albino rabbits. The application sites were graded for indication of skin reactions at 0.5, 24, 48, 72, 168 and 336 hours after test substance removal. There were mild skin irritation reactions in all of the test subjects which cleared by the 336 hour observation. The maximum skin irritation score was 3.0 at the 48 hour observation. The primary skin irritation index was calculated to be 2.58. In accordance with the OPPTS Guidelines, the formulation of the invention would be classified as EPA Toxicity Category IV for dermal effects.

Test VI. Acute Eye Irritation Study in Rabbits

[0127] A formulation of the invention comprising 2-undecanone, soybean oil, polysorbate 20, sorbitan oleate, oil of lemon eucalyptus and denatured alcohol was tested for eye irritation in accordance with OPPTS and OECD Guidelines. The formulation, an amber liquid, was administered into one eye of each of three albino rabbits. The eyes were observed and scored at 1, 24, 48, 72 and 168 hours. There was moderate eye irritation observed in all of the three test subjects which cleared by the 168 hour observation. The maximum group mean score was 14.67/110.0 at the 24 hour observation. In accordance with the OPPTS Guidelines, the formulation of the invention would be classified as Toxicity Category III.

Example 10

[0128] Human Repeat Insult Patch Tests for Skin Irritation and Skin Sensitization Evaluation

[0129] To determine the irritation and sensitization (contact allergy) potential of a test material after repeated application to the skin of human subjects three studies were conducted using the formulations of the invention comprising 2-undecanone, soybean oil, polysorbate 20, sorbitan oleate, oil of lemon eucalyptus and denatured alcohol (e.g., test materials).

Procedures:

[0130] Test materials to be tested under occlusive conditions were placed on an 8-millimeter aluminum Finn Chamber® (Epitest Ltd. Oy, Tuusula, Finland) supported on Scanpor® Tape (Norgesplaster A/S, Kristiansand, Norway) or an 8 mm filter paper coated aluminum Finn Chamber® AQUA supported on a thin flexible transparent polyurethane rectangular film coated on one side with a medical grade acrylic adhesive, consistent with adhesive used in state-of the-art hypoallergenic surgical tapes or a 7 mm IQ-ULTRA® closed cell system which is made of additive-free polyethylene plastic foam with a filter paper incorporated (It is supplied in units of 10 chambers on a hypoallergenic nonwoven adhesive tape; the width of the tape is 52 mm and the length is 118 mm) or other equivalents. Materials to be

tested under semi-occlusive conditions were placed on a test strip with a Rayon/Polypropylene pad or on a 7.5 mm filter paper disc affixed to a strip of hypoallergenic tape (Johnson & Johnson 1 inch First Aid Cloth Tape).

[0131] Test materials to be tested in an open patch were applied and rubbed directly onto the back of the subject. Approximately 0.02-0.05 mL (in case of liquids) and/or 0.02-0.05 gm (in case of solids) of the test material was used for the studies. Liquid test material was dispensed on a 7.5 mm paper disk, which fit in the Finn Chamber.

[0132] Subjects were requested to bathe or wash as usual before arrival at the facility. Patches containing the test material were then affixed directly to the skin of the intrascapular regions of the back, to the right or left of the midline and subjects were dismissed with instructions not to wet or expose the test area to direct sunlight. Patches remained in place for 48 hours after the first application. Subjects were instructed not to remove the patches prior to their 48 hour scheduled visit. Thereafter, subjects were instructed to remove patches 24 hours after application for the remainder of the study.

[0133] This procedure was repeated until a series of nine (9) consecutive, 24-hour exposures had been made three (3) times a week for three (3) consecutive weeks. Prior to each reapplication, the test sites were evaluated by trained laboratory personnel. Following a 10-14 day rest period a retest/challenge dose was applied once to a previously unexposed test site.

[0134] Test sites were evaluated by trained laboratory personnel 48 and 96 hours after application.

[0135] In the event of an adverse reaction, the area of erythema and edema were measured. Edema was estimated by the evaluation of the skin with respect to the contour of the unaffected normal skin. Subjects were instructed to report any delayed reactions that might occur after the final reading.

[0136] Scoring scale and definition of symbols shown below are based on the scoring scheme according to the International Contact Dermatitis Research Group scoring scale [Rietschel, R. L., Fowler, J. F., Ed., Fisher's Contact Dermatitis (fourth ed.). Baltimore, Williams & Wilkins, 1995] listed below:

[0137] 0 no reaction (negative)

[0138] 1 erythema throughout at least ¾ of patch area

[0139] 2 erythema and induration throughout at least ¾ of patch area

[0140] 3 erythema, induration and vesicles

[0141] 4 erythema, induration and bullae

[0142] D Site discontinued

[0143] Dc Subject discontinued voluntarily

[0144] DcI Subject discontinued per Investigator

Study I.

[0145] Formulation: 2-undecanone, soybean oil, polysorbate 20, sorbitan oleate, oil of lemon eucalyptus and denatured alcohol.

Population Demographics

[0146]

Number of subjects enrolled	66
Number of subjects completing study	59
Age Range	19-65
Sex	
Male	4
Female	55
Fitzpatrick Skin Type*	
1 - always burn, does not tan	0
2 - burn easily, tan slightly	22
3 - burn moderately, tan progressively	8
4 - burn a little, always tan	17
5 - rarely burn, tan intensely	12
6 - never burn, tan very intensely	0

*Agache P., Hubert P.. *Measuring the skin*. Springer-Verlag Berlin Heidelberg, 2004, (p. 473, table 48.1)

[0147] Results: No adverse reactions of any kind were reported during the course of this study. There were thirty-five (35) subjects with a Grade 1 reaction and four (4) subjects with a Grade 1 delayed reaction to the positive control (2.0% Sodium Lauryl Sulfate Solution).

[0148] No subjects showed any signs of reaction to the negative control (DI Water).

Study II.

[0149] Formulation: 2-undecanone, soybean oil, polysorbate 20, sorbitan oleate, oil of lemon eucalyptus and denatured alcohol.

Population Demographics:

[0150]

Number of subjects enrolled	55
Number of subjects completing study	55
Age Range	18-64
Sex	
Male	9
Female	46
Fitzpatrick Skin Type*	
1 - always burn, does not tan	0
2 - burn easily, tan slightly	0
3 - bum moderately, tan	55
4 - bum a little, always tan	0
5 - rarely burn, tan intensely	0
6 - never burn, tan very intensely	0

*Agache P., Hubert P.. *Measuring the skin*. Springer-Verlag Berlin Heidelberg, 2004, (p. 473, table 48.1)

[0151] Results: No adverse reactions of any kind were reported during the course of this study. There were Ten (10) subjects with a Grade 1 reaction to the positive control (2.0% Sodium Lauryl Sulfate Solution). No subjects showed any signs of reaction to the negative control (DI Water).

Study III

[0152] Formulation: 2-undecanone, soybean oil, polysorbate 20, sorbitan oleate, oil of lemon eucalyptus and denatured alcohol.

Population Demographics:

[0153]

Number of subjects enrolled	55
Number of subjects completing study	54
Age Range	19-63
Sex	
Male	5
Female	49
Fitzpatrick Skin Type*	
1 - always burn, does not tan	0
2 - burn easily, tan slightly	0
3 - burn moderately, tan progressively	54
4 - burn a little, always tan	0
5 - rarely burn, tan intensely	0
6 - never burn, tan very intensely	0

*Agache P., Hubert P.. *Measuring the skin.* Springer-Verlag Berlin Heidelberg, 2004, (p. 473, table 48.1)

[0154] Results: No adverse reactions of any kind were reported during the course of this study. Two subjects showed Grade 1 and two subjects showed Grade 2 reaction to the positive control (2.0% Sodium Lauryl Sulfate Solution). No subjects showed any signs of reaction to the negative control (DI Water).

[0155] The foregoing is illustrative of the present invention, and is not to be construed as limiting thereof. The invention is defined by the following claims, with equivalents of the claims to be included therein.

That which is claimed is:

1. A monophasic formulation comprising:

2-undecanone in an amount of about 5% to about 20% by volume in the formulation;

at least one hydrophobic base in an amount of about 20% to about 65% by volume in the formulation;

at least one alcohol in an amount of about 5% to about 30% by volume in the formulation; and

at least one emulsifier in an amount of about 5% to about 15% by volume in the formulation.

2. The monophasic formulation of claim **1**, further comprising at least one of oil of lemon eucalyptus (OLE), p-menthane-3,8-diol, and/or geraniol, optionally in an amount of about 5% to about 15%.

3. The monophasic formulation of claim **1**, wherein the at least one hydrophobic base is a branched hydrocarbon, an unbranched hydrocarbon, a hydrophobic polymer, a fatty acid, a fatty acid ester, a monoglyceride, a diglyceride, and/or a triglyceride.

4. The monophasic formulation of claim **1**, wherein the at least one hydrophobic base is a plant-based oil, optionally wherein the plant-based oil is avocado oil, borage oil, canola (rapeseed) oil, castor oil, chamomile oil, coconut oil, corn oil, cottonseed oil, rapeseed oil, evening primrose oil, safflower oil, sunflower oil, soybean oil, sweet almond, palm oil, palm kernel oil, arctium lappa seed oil, sesame oil, borgo officialis seed oil, brassica campestris oleifera oil, brevoortia oil, bubulum oil, cistus ladaniferus oil, elaeis guineensis oil, almond oil, pine oil, olive oil, peanut oil, flax seed oil, wheat germ oil, hemp oil, jojoba oil, linseed oil, macadamia oil, neem oil, argan oil, grape seed oil, thistle oil, and/or algal oil and/or any combination thereof.

5. The monophasic formulation of claim **1**, wherein the hydrophobic base is soybean oil, cottonseed oil and/or coconut oil.

6. The monophasic formulation of claim **1**, wherein the at least one alcohol is ethanol, isopropyl alcohol, t-butyl alcohol, propyl alcohol, benzyl alcohol, and/or denatured alcohol.

7. The monophasic formulation of claim **1**, wherein the at least one emulsifier is polysorbate 20, sorbitan oleate, potassium sorbate, and/or sodium benzoate.

8. A method of repelling an insect pest or a tick, comprising:

applying the monophasic formulation of claim **1** to a subject or a substrate in an amount effective to repel the insect or tick.

9. The method of claim **8**, wherein the insect is a mosquito, sand fly, filth fly, chigger, flea, black fly, deer fly, horse fly, lice (e.g., body lice, head lice), roach, ant, and/or fire ant.

10. The method of claim **8**, wherein about 90% to about 100% of the insects and/or ticks are repelled and/or the insects and/or ticks are repelled for up to about 7 hours after application.

11. The method of claim **8**, wherein the subject is a human and/or the substrate is fabric, cotton products, clothing, wrist bands, bedding, linens, towels, curtains, draperies, wood products, furniture, carpet, floors, walls, baseboards, netting, paper, water, trash containers, dumps, tents, paint, plants, and/or soil.

12. A product infused with the monophasic formulation of claim **1**.

13. The product of claim **12**, wherein the product is a textile, a plastic, latex, rubber, cellulose, a wick, a candle, paint, a cosmetic, a lotion, a shampoo, a conditioner, and/or a mousse.

14. The product of claim **13**, wherein the textile is clothing, bedding, linens, towels, curtains, draperies, tents, and/or netting.

15. A monophasic formulation comprising:

2-undecanone that repels insects and ticks in an amount of about 5% to about 10% by volume in the formulation;

at least one hydrophobic base in an amount of about 45% to about 55% by volume in the formulation;

at least one alcohol in an amount of about 10% to about 15% by volume in the formulation;

at least one emulsifier in an amount of about 8% to about 12% by volume in the formulation; and

oil of lemon eucalyptus (OLE) in an amount of about 8% to about 12% by volume in the formulation.

16. The monophasic formulation of claim **15**, wherein the hydrophobic base is soybean oil.

17. The monophasic formulation of claim **15**, wherein the at least one alcohol is ethanol, isopropyl alcohol and/or denatured alcohol.

18. The monophasic formulation of claim **15**, wherein the at least one emulsifier is polysorbate 20 and sorbitan oleate.

19. A method of repelling an insect pest or a tick, comprising:

applying the monophasic formulation of claim **15** to a subject or a substrate in an amount effective to repel the insect or tick.

20. The method of claim **19**, wherein the insect is a mosquito, sand fly, filth fly, chigger, flea, black fly, deer fly, horse fly, lice (e.g., body lice, head lice), roach, ant, and/or fire ant.

21. The method of claim **19**, wherein about 90% to about 100% of the insects and/or ticks are repelled and/or the insects and/or ticks are repelled for up to about 7 hours after application.

22. The method of claim **19**, wherein the subject is a human and/or wherein the substrate is fabric, cotton products, clothing, wrist bands, bedding, linens, towels, curtains, draperies, wood products, furniture, carpet, floors, walls, baseboards, netting, paper, water, trash containers, dumps, tents, paint, plants, and/or soil.

23. A product infused with the monophasic formulation of claim **15**.

24. The product of claim **23**, wherein the product is a textile, a plastic, latex, rubber, cellulose, a wick, a candle, paint, a cosmetic, a lotion, a shampoo, a conditioner, and/or a mousse.

25. The product of claim **24**, wherein the textile is clothing, bedding, linens, towels, curtains, draperies, tents, and/or netting.

* * * * *

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(54) **INSECT AND TICK REPELLENT FORMULATIONS AND METHODS OF USE THEREOF**

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PCT Pub. Date: **Jul. 4, 2019**

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(58) **Field of Classification Search**
CPC combination set(s) only.
See application file for complete search history.

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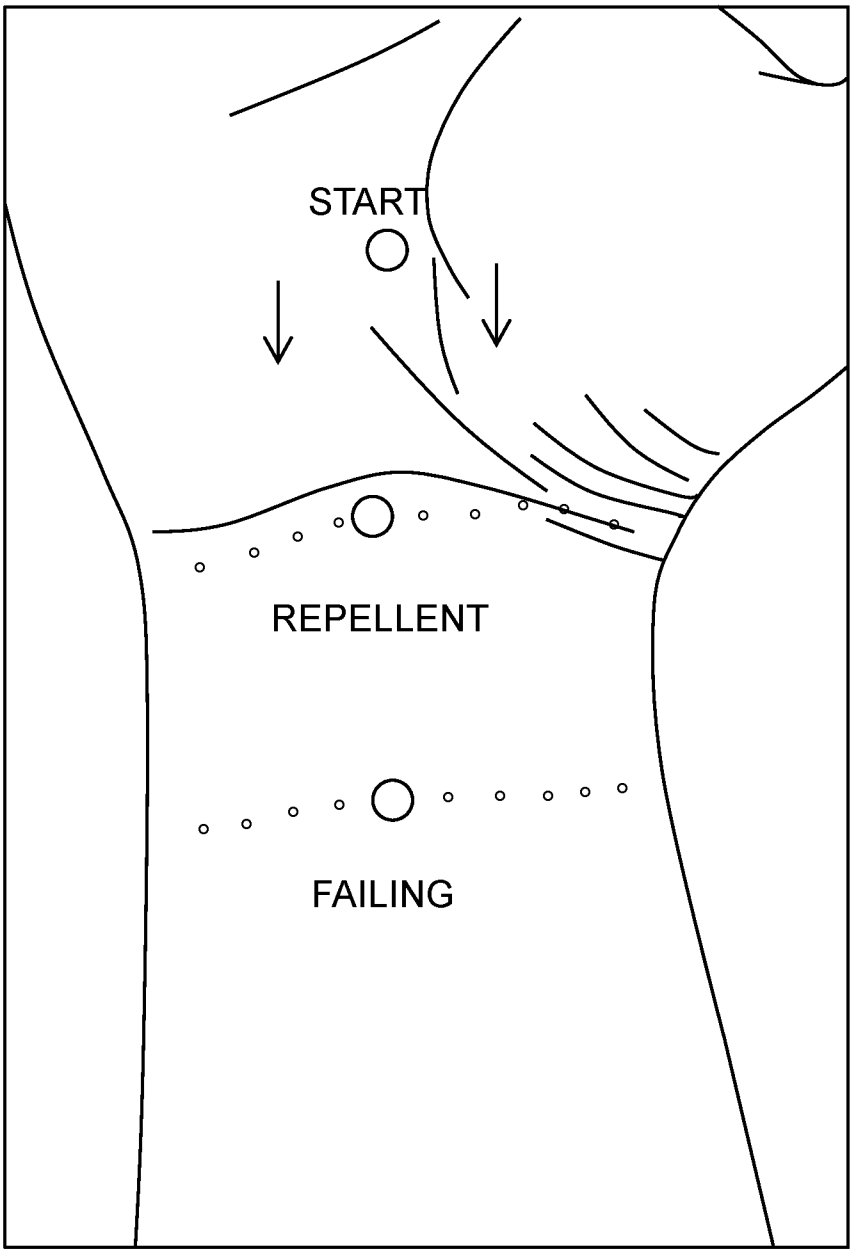
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(57) **ABSTRACT**

The invention relates to a formulation comprising: 2-undecanone in an amount of about 5% to about 50%; at least one hydrophobic base in an amount of about 5% to about 65%; at least one alcohol in an amount of about 5% to about 50%; and at least one emulsifier in an amount of about 5% to about 15% and methods of repelling insects and ticks by applying to a subject or a substrate a formulation of the invention.

20 Claims, 1 Drawing Sheet


START
REPELLENT
FAILING

INSECT AND TICK REPELLENT FORMULATIONS AND METHODS OF USE THEREOF

STATEMENT OF PRIORITY

This application claims the benefit, under 35 U.S.C. § 119 (e), of U.S. Provisional Application No. 62/611,178 filed on Dec. 28, 2017, the entire contents of which is incorporated by reference herein.

FIELD OF THE INVENTION

The present invention concerns methods and formulations for repelling insects and ticks.

BACKGROUND OF THE INVENTION

Insect and tick repellants are widely used throughout the United States and throughout the world. In some regions, the use of repellants is important for avoiding or reducing the occurrence of diseases carried by insects and ticks. For example, the Centers for Disease Control (CDC) receives nearly 10,000 reports of Lyme disease (transmitted by deer ticks) and 1,000 reports of encephalitis (transmitted by mosquitoes) annually.

Currently, the most common repellent is N,N-diethyl-meta-toluamide (DEET). DEET was designed to be applicable to the skin of subjects, and is designed to repel rather than kill insects and ticks. Although in use for some time, concern has recently emerged about the potential toxicity of DEET to children. Recently the US Environmental Protection Agency (EPA) determined that it would no longer allow child safety claims on labels for DEET-containing products.

Improved insect and tick repellants useful for all persons are needed.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** provides photograph of a representative arm with descriptive labels to show the set-up for testing tick foraging. The labels were not present during actual testing.

SUMMARY OF THE INVENTION

A first aspect of the invention provides a formulation comprising: 2-undecanone in an amount of about 5% to about 50% by volume; at least one hydrophobic base in an amount of about 5% to about 65% by volume; at least one alcohol in an amount of about 5% to about 50% by volume; and at least one emulsifier in an amount of about 5% to about 15% by volume.

A second aspect provides a formulation comprising: 2-undecanone in an amount of about 5% to about 30% by volume; at least one hydrophobic base in an amount of about 25% to about 40% by volume; at least one alcohol in an amount of about 25% to about 40% by volume; and at least one emulsifier in an amount of about 5% to about 15% by volume.

A third aspect of the invention provides a formulation comprising: 2-undecanone in an amount of about 10% to about 25% by volume; at least one hydrophobic base in an amount of about 25% to about 35% by volume; at least one alcohol in an amount of about 25% to about 40% by volume; and at least one emulsifier in an amount of about 5% to about 15% by volume.

A fourth aspect of the invention provides a method of repelling an insect pest or a tick, comprising: applying to a subject a formulation of the present invention in an amount effective to repel the insect or tick.

DETAILED DESCRIPTION

The present invention now will be described hereinafter with reference to the accompanying drawings and examples, in which embodiments of the invention are shown. This description is not intended to be a detailed catalog of all the different ways in which the invention may be implemented, or all the features that may be added to the instant invention. For example, features illustrated with respect to one embodiment may be incorporated into other embodiments, and features illustrated with respect to a particular embodiment may be deleted from that embodiment. Thus, the invention contemplates that in some embodiments of the invention, any feature or combination of features set forth herein can be excluded or omitted. In addition, numerous variations and additions to the various embodiments suggested herein will be apparent to those skilled in the art in light of the instant disclosure, which do not depart from the instant invention. Hence, the following descriptions are intended to illustrate some particular embodiments of the invention, and not to exhaustively specify all permutations, combinations and variations thereof.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. The terminology used in the description of the invention herein is for the purpose of describing particular embodiments only and is not intended to be limiting of the invention.

All publications, patent applications, patents and other references cited herein are incorporated by reference in their entireties for the teachings relevant to the sentence and/or paragraph in which the reference is presented.

Unless the context indicates otherwise, it is specifically intended that the various features of the invention described herein can be used in any combination. Moreover, the present invention also contemplates that in some embodiments of the invention, any feature or combination of features set forth herein can be excluded or omitted. To illustrate, if the specification states that a composition comprises components A, B and C, it is specifically intended that any of A, B or C, or a combination thereof, can be omitted and disclaimed singularly or in any combination.

As used in the description of the invention and the appended claims, the singular forms "a," "an" and "the" are intended to include the plural forms as well, unless the context clearly indicates otherwise.

Also, as used herein, "and/or" refers to and encompasses any and all possible combinations of one or more of the associated listed items, as well as the lack of combinations when interpreted in the alternative ("or").

The term "about," as used herein when referring to a measurable value such as a dosage or time period and the like refers to variations of ±10%, ±5%, ±1%, ±0.5%, or even ±0.1% of the specified amount.

As used herein, phrases such as "between X and Y" and "between about X and Y" should be interpreted to include X and Y. As used herein, phrases such as "between about X and Y" mean "between about X and about Y" and phrases such as "from about X to Y" mean "from about X to about Y."

The term "comprise," "comprises" and "comprising" as used herein, specify the presence of the stated features,

integers, steps, operations, elements, and/or components, but do not preclude the presence or addition of one or more other features, integers, steps, operations, elements, components, and/or groups thereof.

As used herein, the transitional phrase "consisting essentially of" means that the scope of a claim is to be interpreted to encompass the specified materials or steps recited in the claim and those that do not materially affect the basic and novel characteristic(s) of the claimed invention. Thus, the term "consisting essentially of" when used in a claim of this invention is not intended to be interpreted to be equivalent to "comprising."

As used herein, the terms "increase," "increasing," "increased," "enhance," "enhanced," "enhancing," and "enhancement" (and grammatical variations thereof) describe an elevation of at least about 25%, 50%, 75%, 100%, 150%, 200%, 300%, 400%, 500% or more as compared to a control.

As used herein, the terms "reduce," "reduced," "reducing," "reduction," "diminish," and "decrease" (and grammatical variations thereof), describe, for example, a decrease of at least about 5%, 10%, 15%, 20%, 25%, 35%, 50%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 100% as compared to a control. In particular embodiments, the reduction can result in no or essentially no (i.e., an insignificant amount, e.g., less than about 10% or even 5%) detectable activity or amount.

The present invention provides monophasic formulations comprising undecanone for use in repelling insects and/or ticks. Thus, in some embodiments, the formulations of the invention are not in the form of emulsions but are in a single phase.

In some embodiments, the formulations may be in the form of an emulsion that is homogeneous.

In some embodiments, the formulations of the invention may be used to repel animals generally including, but not limited to, insects, ticks, spiders, hunting spiders, and scorpions.

Notably, 2-undecanone alone is not an effective insect or tick repellent for at least the reasons that it evaporates too fast and its duration of repellency is less than about 30 min. The formulations of the present invention comprising 2-undecanone, at least one hydrophobic base (for example, a seed oil), at least one alcohol, and at least one emulsifier provide significant repellency lasting over many hours after application to a subject.

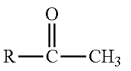
Accordingly, in some embodiments, the present invention provides a formulation comprising: 2-undecanone in an amount of about 5% to about 50% by volume (e.g., about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 50%, or any range or value therein); at least one hydrophobic base in an amount of about 5% to about 65% by volume (e.g., about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 50%, 55%, 60%, 65%, or any range or value therein); at least one alcohol in an amount of about 5% to about 50% by volume (e.g., about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 50%, or any range or value therein); and at least one emulsifier(i.e., total amount of all emulsifiers) in an amount of about 5% to about 15% by volume (e.g., about 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, or any range or value therein). In some embodiments, the total amount of hydrophobic base in the formulation may be in a range from about 5%-65% by volume of the formulation. In some embodiments, the total amount of alcohol in the formulation may be in a range from about 5% to about 50% by volume of the formulation. In some embodiments, the total amount of emulsifier in the formulation may be in a range from about 5% to about 15% by volume of the formulation.

In some embodiments, a formulation is provided comprising: 2-undecanone in an amount of about 5% to about 30% by volume (e.g., about 5%, 10%, 15%, 20%, 25%, 30%, or any range or value therein); at least one hydrophobic base in an amount of about 25% to about 40% by volume (e.g., about 25%, 26%, 27%, 28%, 29%, 30%, 13%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, or any range or value therein); at least one alcohol in an amount of about 25% to about 40% by volume (e.g., about 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40% by volume, or any range or value therein); and at least one emulsifier (i.e., total amount of all emulsifiers) in an amount of about 5% to about 15% by volume or about 5% to about 15% by volume (e.g., about 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, or any range or value therein). In some embodiments, the total amount of hydrophobic base in the formulation may be in a range from about 25%-40% by volume of the formulation. In some embodiments, the total amount of alcohol in the formulation may be in a range from about 25% to about 40% by volume of the formulation. In some embodiments, the total amount of emulsifier in the formulation may be in a range from about 5% to about 15% by volume of the formulation.

In some embodiments, the present invention provides a formulation comprising: 2-undecanone in an amount of about 10% to about 25% by volume (e.g., about 10%, 15%, 20%, 21%, 22%, 23%, 24%, 25%, or any range or value therein; e.g., about 12% to about 16%, about 12% to about 17%, about 13% to about 16%, about 13% to about 17%, about 14% to about 16% by volume, or any range or value therein); at least one hydrophobic base in an amount of about 25% to about 35% by volume (e.g., about 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35%, or any range or value therein; e.g., about 26% to about 33%, about 26% to about 35%, about 28% to about 32%, about 28% to about 33%, about 28% to about 35%,%, or any range or value therein); at least one alcohol in an amount of about 25% to about 40% by volume (e.g., about 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, or any range or value therein; e.g., about 30% to about 38%, about 30% to about 40%, about 32% to about 37%, about 32% to about 38%, about 33% to about 37%, about 33% to about 36%, or any range or value therein); and at least one emulsifier (i.e., total amount of all emulsifiers) in an amount of about 5% to about 15% by volume or about 5% to about 15% by volume (e.g., about 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, or any range or value therein; e.g., about 7% to about 12%, about 7% to about 13%, about 7% to about 14%, about 8% to about 11%, about 8% to about 12%, about 8% to about 13%, or any range or value therein). In some embodiments, a formulation is provided comprising 2-undecanone in an amount of about 10% to about 20% by volume (e.g., about 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, or any range or value therein); at least one hydrophobic base in an amount of about 30% to about 40% by volume (e.g., about 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, or any range or value therein); at least one alcohol in an amount of about 30% to about 40% by volume (e.g., about 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, or any range or value therein); and at least one emulsifier (i.e., total amount of all emulsifiers) in an amount of about 5% to about 15% by volume (e.g., about 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, or any range or value therein). In some embodiments, the total amount of hydro-

phobic base in the formulation may be in a range from about 25%-35% by volume of the formulation or about 30% to about 40% by volume of the formulation. In some embodiments, the total amount of alcohol in the formulation may be in a range from about 25% to about 40% by volume of the formulation or about 30% to about 40% by volume of the formulation. In some embodiments, the total amount of emulsifier in the formulation may be in a range from about 5% to about 15% by volume of the formulation.

2-undecanone (also known as methyl nonyl ketone) comprises the structure of



(I)

wherein R is C_9.

The formulations of the invention comprise a hydrophobic base. "Hydrophobic base" as used herein refers to a natural and/or synthetic fat, oil, and/or the like. Any suitable hydrophobic base may be used in a formulation of the present invention. In certain embodiments of the present invention, a formulation may comprise two or more hydrophobic bases, such as, but not limited to, 2, 3, 4, 5, or more hydrophobic bases. Exemplary hydrophobic bases include, but are not limited to, branched and unbranched hydrocarbons, hydrophobic polymers, fatty acids, fatty acid esters, monoglycerides, diglycerides, and/or triglycerides. A hydrophobic base may be partially or wholly saturated and/or unsaturated.

Additional exemplary hydrophobic bases may include, but are not limited to plant and/or vegetable oils, animal oils, and/or mineral oil. A plant-based oil may be any oil obtained from a plant or plant part (e.g., oil from seeds, flowers, fruit, leaves, bark, roots, etc). Exemplary plant oils include avocado oil, borage oil, canola (rapeseed) oil, castor oil, chamomile oil, coconut oil, corn oil, cottonseed oil, rapeseed oil, evening primrose oil, safflower oil, sunflower oil, soybean oil, sweet almond, palm oil, palm kernel oil, *arctium lappa* seed oil, sesame oil, borgo officialis seed oil, *brassica campestris* oleifera oil, brevoortia oil, bubulum oil, cistus ladaniferus oil, *Elaeis guineensis* oil, almond oil, pine oil, olive oil, peanut oil, flax seed oil, wheat germ oil, hemp oil, jojoba oil, linseed oil, macadamia oil, neem oil, argan oil, grape seed oil, thistle oil, and/or algal oil and/or any combination thereof.

In some embodiments, the molecular weight of a hydrophobic base useful with this invention may range from about 100 g/mol to about 1000 g/mol. Thus, in some embodiments, the molecular weight of a hydrophobic base useful with this invention may range from about 100 g/mol to about 750 g/mol, about 150 g/mol to about 750 g/mol, about 150 g/mol to about 500, about 150 g/mol to about 300 g/mol, and any range or value therein.

In some embodiments, a hydrophobic base may be a 16 carbon or 18 carbon compound or a combination thereof. In some embodiments, a hydrophobic base may be about 10-20% saturated. In some embodiments, a hydrophobic base may be about 75-90% unsaturated. In some embodiments, a hydrophobic base may have a density of about 0.91 to 0.93 g/cm3 between the temperatures of 15° C. and 25° C.

In some embodiments, a formulation of the invention comprises at least one hydrophobic base that is a plant-based oil. In some embodiments, the at least one plant-based oil of a formulation of the invention may comprise, consist essentially of, or consist of soybean oil. In some embodiments, the at least one seed oil of a formulation of the invention may comprise, consist essentially of, or consist of cottonseed oil. In some embodiments, the at least one seed oil of a formulation of the invention may comprise, consist essentially of coconut oil. In some embodiments, the at least one seed oil of a formulation of the invention may comprise, consist essentially of, or consist of soybean oil and cottonseed oil. In some embodiments, the at least one seed oil of a formulation of the invention may comprise, consist essentially of, or consist of soybean oil and coconut oil. In some embodiments, the at least one seed oil of a formulation of the invention may comprise, consist essentially of, or consist of cottonseed oil and coconut oil. In some embodiments, the at least one seed oil of a formulation of the invention may comprise, consist essentially of, or consist of soybean oil, cottonseed oil and coconut oil.

A formulation of the invention comprises at least one alcohol. Non-limiting examples of alcohols useful with the invention include ethanol, isopropyl alcohol, t-butyl alcohol, propyl alcohol, benzyl alcohol or any combination thereof. In some embodiments, the alcohol may come from denatured alcohol and/or vodka. In some embodiments, the at least one alcohol of a formulation of the invention may comprise, consist essentially of, or consist of ethanol. In some embodiments, the at least one alcohol of a formulation of the invention may comprise, consist essentially of, or consist of denatured ethanol. In some embodiments, the at least one alcohol of a formulation of the invention may comprise, consist essentially of, or consist of isopropyl alcohol. In some embodiments, the at least one alcohol of a formulation of the invention may comprise, consist essentially of, or consist of t-butyl alcohol. In some embodiments, the at least one alcohol of a formulation of the invention may comprise, consist essentially of, or consist of propyl alcohol. In some embodiments, the at least one alcohol of a formulation of the invention may comprise, consist essentially of, or consist of benzyl alcohol. In some embodiments, the at least one alcohol of a formulation of the invention may comprise, consist essentially of, or consist of ethanol and isopropyl alcohol. In some embodiments, the at least one alcohol of a formulation of the invention may comprise, consist essentially of, or consist of ethanol, isopropyl alcohol and denatured alcohol.

Any denatured alcohol may be used in a formulation of the invention. In some embodiments, a "specially denatured alcohol" (SDA) may be used. In some embodiments, the denatured alcohol can be 95% ethanol and 5% isopropanol by volume. In some embodiments, the denatured alcohol can be 90% ethanol and 10% methanol by volume.

In some embodiments, a formulation of the invention comprises an equal amount of alcohol to hydrophobic base. In some embodiments, a formulation of the invention comprises about 3%-10% more alcohol than hydrophobic base (e.g., 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10% vol./vol. more alcohol than hydrophobic base) (e.g., about 40% alcohol to about 30% hydrophobic base, about 40% alcohol to about 35% hydrophobic base, about 38% alcohol to about 30% hydrophobic base, about 35% alcohol to about 32% hydrophobic base, about 35% alcohol to about 30% hydrophobic base, about 35% alcohol to about 25% hydrophobic base, about 30% alcohol to about 25% hydrophobic base, about 34% alcohol to about 30% hydrophobic base and the like). In some embodiments, a formulation of the invention comprises about two times the amount of hydrophobic base to alcohol (e.g., about 30% hydrophobic base to about 15%

alcohol, about 40% hydrophobic base to about 20% alcohol, about 50% hydrophobic base to about 25% alcohol; about 60% hydrophobic base to about 30% alcohol, and the like).

A formulation of the invention further comprises emulsifiers to assist in maintaining a stable monophasic system. Non-limiting examples of an emulsifier include polysorbate 20, sorbitan oleate, potassium sorbate, sodium benzoate, polysorbate 80, Tween 20, Tween 21, Tween 40, Tween 60, Tween 61, Tween 80, Tween 81, Tween 85; and/or sodium dodecyl sulfate (SDS). In some embodiments, at least one an emulsifier a formulation of the invention may comprise, consist essentially of, or consist of polysorbate 20, sorbitan oleate, potassium sorbate, and/or sodium benzoate. In some embodiments, the at least one emulsifier of the formulation may comprise, consist essentially of, or consist of polysorbate 20. In some embodiments, the at least one emulsifier of the formulation may comprise, consist essentially of, or consist of sorbitan oleate. In some embodiments, the at least one emulsifier of the formulation may comprise, consist essentially of, or consist of polysorbate 20 and sorbitan oleate.

In some embodiments, the formulations of the invention described herein may further comprise at least one of oil of lemon eucalyptus (OLE), p-methane-3,8-diol, and/or geraniol in an amount of about 5% to about 15% by volume (e.g., about 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, or any range or value therein; e.g., about 7% to about 12%, about 7% to about 13%, about 7% to about 14%, about 8% to about 11%, about 8% to about 12%, about 8% to about 13%, or any range or value therein). In some embodiments, the formulation may comprise geraniol in an amount of about 5% to about 15%. In some embodiments, the formulation may comprise OLE in an amount of about 5% to about 15%. In some embodiments, a formulation of the invention may comprise p-methane-3,8-diol in an amount of about 5% to about 15%. In some embodiments, a formulation of the invention may comprise geraniol and OLE in an amount of about 5% to about 15%. Thus, in exemplary embodiments, a formulation of the invention may comprise 2-undecanone in an amount of about 5% to about 50%, about 5% to about 30%, about 10% to about 20% or about 12% to about 18% by volume, or any range or value therein; at least one hydrophobic base in an amount of about 5% to about 65%, about 25% to about 40%, about 25% to about 35%, or about 27% to about 33% by volume, or any range or value therein; at least one alcohol in an amount of about 5% to about 50%, about 25% to about 40%, about 30% to about 40% or about 32% to about 38% by volume, or any range or value therein; at least one emulsifier in an amount of about 5% to about 15%, about 6% to about 13%, about 7% to about 12% or about 8% to about 12% by volume, or any range or value therein; and at least one of OLE, p-methane-3,8-diol, and/or geraniol in an amount of about 5% to about 15%, about 6% to about 13%, about 7% to about 12% or about 8% to about 12% by volume, or any range or value therein. In some embodiments, a formulation of the invention may comprise 2-undecanone in an amount of about 12% to about 18% by volume; at least one hydrophobic base in an amount of about 28% to about 33% by volume, at least one alcohol in an amount of about 30% to about 35% by volume; at least one emulsifier in an amount of about 8% to about 12% by volume; and at least one of OLE, p-methane-3,8-diol, and/or geraniol in an amount of about 8% to about 12% by volume. In some embodiments, the formulations of the invention described herein comprise OLE in an amount of about 5% to about 15%, about 8% to about 12% or about 8% to about 13%, about 9% to about 12% by volume, and any range or value therein.

In some embodiments, a formulation of the invention may be anhydrous. In some embodiments, a formulation of the invention may comprise water in an amount less than 0.25% vol/vol. In some embodiments, a formulation of the invention may comprise water in a range from about 0.25% vol./vol. to about 50% vol./vol.

The formulations of the invention may be used to repel animals including, but not limited to, insects and ticks.

Accordingly, in some embodiments, the present invention provides a method of repelling an insect or a tick, comprising applying to a subject a formulation of the invention as describe herein in an amount effective to repel an insect or a tick. In some embodiments, an effective amount of a formulation of the invention can comprise by volume about 5% to about 50%, about 5% to about 40%, about 5% to about 30%, about 5% to about 20%, about 5% to about 17.5%, about 5% to about 15%, about 10% to about 50%, about 10% to about 40%, about 10% to about 30%, about 10% to about 20%, about 10% to about 17.5%, about 12% to about 17%, 13% to about 17%, 14% to about 16%, about 15% to about 50%, about 15% to about 40%, about 15% to about 30%, about 15% to about 20%, about 15% to about 17.5%, about 15% to about 18%, about 20% to about 50%, about 20% to about 40%, about 20% to about 30%, about 30% to about 50%, about 30% to about 40%, about 30% to about 50%, about 5%, about 6%, about 7%, about 8%, about 9%, about 10%, about 10.5%, about 11%, about 11.5%, about 12%, about 12.5%, about 13%, about 13.5%, about 14%, about 14.5%, about 15%, about 15.5%, about 16%, about 16.5%, about 17%, about 17.5%, about 18%, about 18.5%, about 19%, about 19.5%, about 20%, about 20.5%, about 21%, about 22%, about 23%, about 24%, about 25%, about 26%, about 27%, about 28%, about 29%, about 30%, about 35%, about 40%, about 45%, or about 50% of 2-undecanone, and any range or value therein.

In some embodiments, application of a formulation of the invention may be repeated as needed. Thus, in some embodiments, the application of the formulation may be repeated every six hours, every 12 hours, every 24 hours, every other day, every three days, once a week, and the like, as needed.

In some embodiments, the formulations of the invention may be used to repel animals generally including, but not limited to, insects, ticks, spiders, hunting spiders, and/or scorpions. In some embodiments, the formulations of the invention may be used for repelling insects and ticks, wherein the insect may be, for example, a mosquito, sand fly, filth fly, chigger, flea, black fly, deer fly, horse fly, lice (e.g., body lice, head lice), roach, ant, fire ant, gnat, no-see urn, and/or chigger, and the like.

The term "mosquito" as used herein refers to any type of mosquito (e.g., *Anopheles, Aedes*, and *Culex*), including but not limited to Tiger mosquitoes, *Aedes aboriginis, Aedes Aegypti, Aedes, albopictus, Aedes cantator, Aedes sierrensis, Aedes sollicitans, Aedes squamiger, Aedes sticticus, Aedes vexans, Anopheles quadrimaculatus, Culex pipiens*, and/or *Culex quinquefaxciatus.*

The term "tick" as used herein refer to any type of tick, including but not limited to, deer ticks, the American dog tick (*Dermacentor variabilis*), *Ornithodoros parkeri, O. moubata*, and/or *Dermacentor andersoni.*

The term "roach" as used herein refers to any type of roach, including but not limited to the American cockroach (*Periplaneta americana*), German cockroach (*Blattella germanica*), oriental cockroach (*Blatta orientalis*), wood cock-

roach (*Parcoblatta pennsylvanica*), brownbanded cockroach (*Supella longipalpa*), and/or smokybrown cockroach (*Periplaneta fuliginosa*).

In some embodiments, a formulation of the present invention may repel at least about 90% (e.g., at least about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 100%, and any value or range therein) (e.g., percent repellency) of the insects and/or ticks up to about seven hours (e.g., about 1 min, 5 min, 10 min, 15 min, 20 min, 25 min, 30 min, 35 min, 40 min, 45 min, 50 min, 55 min, 1 hr, 1.1 hrs, 1.2 hrs, 1.3 hrs, 1.4 hrs, 1.5 hrs, 1.6 hrs, 1.7 hrs, 1.8 hrs, 1.9 hrs, 2 hrs, 2.1 hrs, 2.2 hrs, 2.3 hrs, 2.4 hrs, 2.5 hrs, 2.6 hrs, 2.7 hrs, 2.8 hrs, 2.9 hrs, 3 hrs, 3.1 hrs, 3.2 hrs, 3.3 hrs, 3.4 hrs, 3.5 hrs, 3.6 hrs, 3.7 hrs, 3.8 hrs, 3.9 hrs, 4 hrs, 4.1 hrs, 4.2 hrs, 4.3 hrs, 4.4 hrs, 4.5 hrs, 4.6 hrs, 4.7 hrs, 4.8 hrs, 4.9 hrs, 5 hrs, 5.1 hrs, 5.2 hrs, 5.3 hrs, 5.4 hrs, 5.5 hrs, 5.6 hrs, 5.7 hrs, 5.8 hrs, 5.9 hrs, 6 hrs, 6.5 hrs, or 7 hrs or any range or value therein) after application of the formulation to a subject. Thus, in some embodiments, a formulation of the invention may be therapeutically effective (e.g., repels about 90% to 100% of insects and ticks) for about 4 hrs to about 7 hrs or more after application to a subject. In some embodiments, a formulation of the present invention may repel at least about 90% to 100% of insects (e.g., mosquitoes) for up to about 4 hrs to about 6 hrs, about 4 hrs to about 5 hrs, about 5 hrs to about 6 hrs, or any range or value therein after application to a subject. In some embodiments, a formulation of the present invention may repel at least about 90% to 100% of ticks for up to about 5 hrs to about 7 hrs, about 5 hrs to about 8 hrs, about 6 hrs to about 7 hrs, about 6 hrs to about 8 hrs, or any range or value therein after application to a subject.

As is well understood, the repellency and duration of repellency for ticks versus insects such as mosquitoes can be different. This is due in part to the fact that odorant receptors on mosquitoes are on the antennae found on the head while those for ticks are on the tarsi (feet) on the front pair of legs. When repellency studies are conducted on mosquitoes, the mosquitoes are flying toward the human subject, detect the repellent that has evaporated from the skin of the human subject into the air some distance from the host (decreasing by 1 divided by distance squared from the repellent origin on the skin), and moving away from the repellent source (the skin). Because the mosquito is moving rapid through space in the direction of the host cues, the chemical repellency (amount of repellent in air and its repellent potency based on its unit of repellent activity per mole) must be high for repellency to occur. In the case of ticks, they are already on the host surface (skin or hair) or on host clothing and potentially moving toward a surface protected by the repellent. In this case, they are essentially walking on their olfactory surface, which comes into almost direct contact with the repellent treated surface. The repellent level in the air away from the host is not important but only that on or near the skin surface. The formulation developed in this invention was designed to provide the maximum duration of repellency for mosquitoes but also at same time provide maximum duration of repellency for ticks. Thus, in some embodiments, tick repellent duration may be longer because the tick detection and response to the repellent is at the repellent source on the skin surface and the duration of exposure to the repellent per unit time is higher since the tick is crawling at a much lower linear rate than mosquitoes that are flying toward the host. The formulation of the invention is designed to provide a longer tick duration than that for mosquitoes than current commercial repellents but at the same time provide a mosquito repellent duration competitive with current commercial mosquito repellents with comparative percentage levels of different active ingredients. Thus, in addition to insect and mosquito repellency, the present invention provides long lasting tick repellency that is advantageous in many locations where ticks may pose a greater human health risk than mosquitoes relative to the transmission of human diseases (for example, in the US and Europe).

In some embodiments, a formulation of the present invention may repel about 90% to about 100%, about 95% to about 98%, about 95% to about 99%, about 95% to about 100%, about 96% to about 98%, about 96% to about 99%, about 96% to about 100%, about 97% to about 98%, about 97% to about 99%, about 97% to about 100%, about 98% to about 99%, about 98 to about 100%, or about 99 to about 100% of the insects and/or ticks and any range or value therein for up to about seven hours after application of the formulation to a subject. In some embodiments, a formulation of the invention may repel about 95% to about 99% of the insects and ticks for up to about six hours after application of the formulation to a subject. In some embodiments, a formulation of the invention may repel about 95% to about 99% of the insects and ticks for up to about five hours after application of the formulation to a subject. In some embodiments, a formulation of the invention may repel about 98% to about 100% of insects and ticks for up to about four hours after application of the formulation to a subject. In some embodiments, a formulation of the invention may repel about 95 to about 99% of the insects and ticks for more than six hours (e.g., 6, 7, 8, 9, 10, 11, 12 hrs or more) after application of the formulation to a subject.

In some embodiments, a formulation of the invention may repel about 90% to about 100% (90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 100%, and any range or value therein) of insects and ticks for up to about 16 weeks (e.g., about 6 hours, 7 hours, 8 hours, 9 hours, 10 hours, 11 hour, 12 hours, 18 hours, 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 1 week, 2 weeks, 3 weeks, 4 weeks, 5 weeks, 6 weeks, 7 weeks, 8 weeks, 9 weeks, 10 weeks, 11 weeks, 12 weeks, 13 weeks, 14 weeks, 15 weeks, 16 weeks or more, or any range or value therein) when applied to a substrate. Thus, in some embodiments, a formulation of the invention applied to a substrate may repel about 90% to about 100%, about 95% to about 98%, about 95% to about 99%, about 95% to about 100%, about 96% to about 98%, about 96% to about 99%, about 96% to about 100%, about 97% to about 98%, about 97% to about 99%, about 97% to about 100%, about 98% to about 99%, about 98 to about 100%, or about 99 to about 100%, or any value or range therein, of insects and/or ticks for up to about 16 weeks and possibly longer after application of the formulation to the substrate.

In some embodiments, a determination of the percent of insects or ticks repelled by the formulation of the invention can be through the use of in an "arm in the cage" test or a "filter paper" test.

Filter paper test. For the filter paper test, a 1 ft×1 ft×1 ft Plexiglas with a stocking sleeve covering one end (the one end that does not contain a Plexiglass side) (e.g., a plastic cage with four sides and a bottom and a top and one side having a stocking sleeve instead of a plastic side). The cage is filled with a sufficient number of mosquitoes (females only) so that when two grade number 1 filter paper discs (a control and a treated disc) (3.5 inches (9 cm) in diameter) are hung on the walls of the cage (on opposite sides of the cage), at least about 10 mosquitoes land per disc when the mosquitoes in the cage are excited into flight by blowing into the cage and then left undisturbed for 1 min. The assay is conducted at 25-27° C. under low light conditions (humidity

is not regulated but typical lab conditions). The environmental conditions are the same throughout the studies.

For each study, one filter paper disc is treated with the repellent (500 microliters per disc) and the other with the same solvent (500 microliters per disc) used to deliver the repellent. To evenly distribute the test compound and control solvent, the filter paper in each case is treated by application of small droplets added evenly over the entire surface of the paper. Filter paper is particularly useful because it allows the droplets to spread out from the point of application by absorption and diffusion within the cellulose disc. Once the filter paper discs are treated, they are laid horizontal on a glass surface on the lab bench at room temperature of about 25° C. plus/minus 2° C. and 60% relative humidity.

At different times after treatment of the paper, the discs are hung in the cage (the control (treated with carrier solvent only) is hung first and the disc carrying the formulations of the invention are hung second. The researcher handling the discs wears powder-free nitrile examination gloves. The process of placing the discs in the cage and also blowing into the cage by the researcher causes the mosquitoes to fly. Once the researcher removes his/her hands, the number of mosquitoes landing and remaining on the disc for more than three seconds is considered not to be repelled and is counted as such. The test is run over a fixed time, e.g., about 1 min) The researcher blows into the cage one additional time at the 30 sec interval into the 1 min observation period. The discs are removed in the same order that they were placed into the test cage by the researcher who is wearing a new pair of clean gloves. Since the discs are present in the cage for no more than about 1 min, any repellent released into the air will have minimum effects on the physiology of the mosquito. Percentage repellency is calculated as the number of mosquitoes landing on the control paper minus the number of mosquitoes landing on the treatment divided by the number of mosquitoes on the control paper times 100. Since the assay takes only 1 min to conduct, multiple compounds can be tested on the same cohort of mosquitoes. Periodically, the assay is checked to determine that no positional bias is occurring in the cage (e.g., the mosquitoes may favor one filter paper versus another due to the position of one of the filter paper discs relative to a light source). Therefore, control experiments are conducted from time to time to determine if both filter paper discs untreated attract approximately the same number of mosquitoes.

Tick repellency filter paper test. Tick repellency was also determined using a filter paper test. In this case, naïve, unfed, host-seeking (as determined by raised forelegs in response to the investigator's breath), mixed sex, adult ticks were used in all trials. Adult ticks (all three species) for use in repellency studies were held in plastic vials at about 28° C., about 75% relative humidity (RH), and a photoperiod of 14 hr light:10 h dark, including dusk and dawn periods (60 min each). Behavioral bioassays were conducted at 25 C, 65% RH, and in complete darkness (except during the approximately 5 seconds to monitor tick distribution).

Ticks were allowed to choose between a treated and untreated filter paper surface. Tests were conducted in 63.6 cm^2 Petri plate lids lined with two 31.8 cm^2 semi-circle pieces of filter paper (Whatman no. 1), each treated with the formulations of the invention and allowed to dry for 3 h at room temperature under a fume hood. At 3 h, six ticks were placed in each arena along the line formed by the junction of treated and untreated papers. Distribution of ticks was recorded every 5 min from 5 to 30 min after the introduction of ticks to test arenas. An untreated control was used to determine the distribution of ticks in the absence of a repellent. Trials were repeated six times.

Arm-in-the cage-test. For the arm in the cage test, the arm of a human subject (treated and untreated) is retained in the cage for 1 min.

A fixed amount of a test compound (e.g., formulation of the invention) (1 ml) is applied to one forearm by a researcher (the observer) who is wearing non-allergenic gloves on both hands. The observer applies the test compound using a pipette with a sterile tip and then spreads and massages the test compound into the skin from the elbow to the wrist on one arm of the subject (i.e., test arm).

At different times after application of the formulations of the invention, the subject applies a non-allergic glove to the hand on the test arm and inserts their arm into a cage of up to 200 female mosquitoes for about 1 min to about 5 min (e.g., about 1, 2, 3, 4, 5 min). A glove is used to cover the hand because the hand is not part of the assay—only the arm is used in the assay. The length of time may be varied depending on the activity of the mosquito or the mosquito species. It is known that different mosquito species and exact age of mosquito as well as unknown environmental and genetic variation in mosquitoes can affect activity (flying, landing and trying to bite). The repellency may be measured at multiple times post-treatment. Thus, the test may be run, for example, at about 4 hr, 5 hr, 6 hr, 7 hr, 8 hr, 9 hr, 10 hr, 11 hr, or 2 hr post-treatment. Between tests, the subject removes and disposes of the glove on the hand of the treated arm. For each time point, a new glove is used.

Mosquitoes that land on the skin for more than 2 seconds are counted as landing and the subject will move their arm in the cage so that the mosquito is not provided the opportunity to bite. An observer will count the number of landings.

The measurement of landings on the untreated arm (control arm) will precede the measurements made on treated arm. The subject will also have a glove on their untreated arm at each testing point, which will be replaced with a new glove for each time point.

The experiment is designed to observe about 200 or more landings on the untreated arm within one min. The mosquitoes are not allowed to take a blood meal and are prevented from doing so by shaking the arm. Any time a mosquito touches the arm, it is counted as a landing; that is, the mosquitoes do not have to land on the skin with their feet. The percentage repelled is determined by subtracting the number of insects, for example, mosquitos, that land on the treated arm from the number of mosquitos that land on the untreated arm and dividing this sum by the number of mosquitos that land on the untreated arm times 100.

The mosquitoes used in all tests are lab reared, disease free, female, and have not fed on blood of any type before the tests are conducted.

Thus, in some embodiments, a formulation of the present invention repels about 90% to about 100%, about 95% to about 98%, about 95% to about 99%, about 95% to about 100%, about 96% to about 98%, about 96% to about 99%, about 96% to about 100%, about 97% to about 98%, about 97% to about 99%, about 97% to about 100%, about 98% to about 99%, about 98 to about 100%, or about 99 to about 100% of the insects and/or ticks about four hours to about 7 hours after application of the formulation to an arm in the arm in the cage test, and any range or value therein. In some embodiments, a formulation of the invention repels about 100% of the insects and ticks about four hours after application of the formulation to an arm in an arm in the cage test. In some embodiments, a formulation of the invention repels about 99% of the insects and ticks about five hours after application of the formulation to an arm in the arm in the cage test.

Subjects to which the formulations of the present invention may be applied include human subjects and other animals (e.g., dogs, cats, horses, cattle). In some embodiments, a formulation is applied to a subject's skin and/or hair. In some embodiments, when the formulation of the invention is applied to a subject's hair the percent and time period of repellency may be greater than for application of the formulation to the subject's skin.

The formulations of the invention may be applied to subjects directly or indirectly, such as by applying the formulation to the skin or hair of the subject, or by applying the formulation to an article worn by or otherwise protecting/shielding the subject. As an example, a formulation of the invention may be applied to netting or screening that protects a subject.

A formulation of the invention may be applied to any substrate for which repelling animals, including insects and ticks is desired.

In some embodiments, a substrate may include, but is not limited to, fabrics (e.g., clothing, towels, linens, bed clothing, draperies, curtains, and the like); cotton products, wrist bands, wood products, furniture (e.g., chairs, mattresses, box springs, etc.), carpets, floors, soil and/or plants, containers, walls, base boards, air, soil, neting, paper, trash containers, dumps, tents, paint, etc.

Application to subjects or substrates may be carried out by any means that transfers the formulation to the subject or substrate, including but not limited to, spraying, misting, spreading, dusting, pouring, dipping, sprinkling, rubbing, fumigating, rolling, atomizing, and the like.

The formulations of the invention may be formulated in a variety of ways, including but not limited to liquids, powders, solids, emulsions, mousses, granules, dusts, a suspension, an aerosol (e.g., powdered, liquid), a shampoo, a conditioner, a creme rinse, a mousse, a foam, a lotion, a gel, a powder (e.g., a dry shampoo), a shampoo, a conditioner, a creme rinse, a paste, or an ointment. The formulations of the invention may be incorporated into or coated onto textiles, plastics, latex, rubber, cellulose, wicks, candles, paint, heat and air dispensers.

The invention will now be described with reference to the following examples. It should be appreciated that these examples are not intended to limit the scope of the claims to the invention, but are rather intended to be exemplary of certain embodiments. Any variations in the exemplified methods that occur to the skilled artisan are intended to fall within the scope of the invention.

EXAMPLES

Example 1. Procedures for Testing Repellent Activity Against the Yellow Fever Mosquito (*Aedes aegypti*), the Asian Tiger Mosquito (*Aedes albopictus*), and *Anopheles gambiae* (a Malaria Vector)

A fixed amount of repellent (1 ml) is applied to one forearm of the subject by a researcher (the observer) wearing non-allergenic gloves on both hands. The observer will apply the repellent using a Gilson pipetter with a sterile tip and then spread and massage the repellent into the skin using the pipette tip as a spreading device spreading the repellent from the elbow to the wrist on one arm of the subject (the test arm).

At different times after application of the repellent up to 12 h, the subject will apply a non-allergic glove to the hand on the test arm and then insert their arm into a cage of 200 female mosquitoes for 1-5 min. The length of time might be different depending on the activity of the mosquito or species; mosquito species and exact age of mosquito as well as unknown environmental and genetic variation in mosquitoes can affect activity (flying, landing and trying to bite). Post treatment measurements are made at 4, 5, 6, 7 and 8 h and possibly 12 h if repellency remains greater than 70% at 8 h. Between tests, the subjects remove and dispose of their glove on the hand of the treated arm. A new glove is used for each time point of testing.

Mosquitoes that land on the skin for more than 1 sec. will be considered ready to bite and the subject will move their arm in the cage so the mosquito leaves the skin to avoid actual biting of the subject. An observer will count the number of landings.

The subject has a glove on their untreated or control arm at each testing point. The insertion of the untreated arm will precede the treated arm and number of landings recorded as described. The glove is removed from the untreated arm immediately after removal from the cage as also will be the case for the treated arm. A new glove is used for each time point.

The study is stopped when percentage repellency is less than 50%. Percentage repellency is calculated based on the number of mosquitoes landing on the untreated arm minus the number landing on the treated arm divided by the number landing on the untreated arm times 100.

The mosquitoes used in the study are lab reared, disease free, adult female, and have not fed on blood of any type before the tests are conducted. Since the mosquitoes are not allowed to bite the subjects, there is also no chance for reactions to mosquito bites.

The mosquitoes are killed by freezing after the testing, autoclaved and disposed in the regular trash.

Example 2

A combination of 2-undecanone with soybean oil and geraniol in the ranges tested provided an increased repellency over 2-undecanone in cotton seed soil and over 2-undecanone in soybean oil using polar solvents like water. In Table 1, the four component formulations were problematic for the following reasons: (i) the product separated into multiple phases when allowed to stand at room temperature; (ii) the product needed to be shaken to reform an emulsion before use (to re-establish a single phase) and as a result of (ii), the product (a) was predicted to have delivery issues as a commercial repellent possibly causing harm to users from the accidental application of neat ingredients and (b) would require specialized development of a new delivery system to apply the repellent because of the chemical degradation of the container parts by the separated 2-undecanone and/or geraniol (which adds to the cost of product development for the delivery system, potentially would result in development challenges that might not be solvable (including issues with having to shake the bottle before use) which is not consistent with industry standards for these type of products); further, the product (iii) was too viscous to be applied by aerosol; (iv) had a poor skin feel—felt oily and was uncomfortable to human test subjects; (v) had a high rate of contact transfer from skin to other materials like clothing that might affect product efficacy for mosquito and tick protection; (vi) did not achieve adequate mosquito repellency 100% at 5 h post treatment; (vii) had a strong, pungent smell which would not be desirable to users and hard to mask with other scents to make a final product with an acceptable odor; and (viii) produced undesirable skin reactions (all in part or whole from list given). Thus, the formulations in Table 1 demonstrated that alcohol could be used to decrease soybean oil content and therefore, improve feel on skin. However, these formulations achieved a repellency similar to that of the plant-based oil only.

TABLE 1

Results of repellency tests for selected formulations

Insect	*Aedes Aegypti*					
Formula Name	Active ingredients	1 hr	2 hr	3 hr	4 hr	6 hr	8 hr
Soybean 1	8% undecanone, 5% geraniol, 35% soybean oil, 52% isopropyl alcohol				*88.5% repellent		
Soybean 2	8% undecanone, 5% geraniol, 50% soybean oil, 37% isopropyl alcohol				*94.5% repellent		
Soybean 3	8% undecanone, 5% geraniol, 65% soybean oil, 22% isopropyl alcohol					*100% repellent	
Soybean 4	8% undecanone, 5% geraniol, 87% soybean oil					*100% repellent	

*200 mosquitoes in the cage, calculation of percentage repellency based on number of landings on treated arm versus untreated arm (see example 1 for calculation details); undecanone = 2-undecanone

Example 3

Addition of alcohol breaks the heavy oily feel of the formulation and reduces the ease of transfer of the repellent from treated skin to items like clothing that might come into contact with treated skin. While, Table 2 included a formulation with a different alcohol to plant-based oil concentration than those in Table 1, the negative aspects of the four component system discussed above in Example 2 were still present, including the separation of the formulation into different phases when left standing. Further, a higher level of plant-based oil was still needed to achieve the desired repellency; however, this higher amount of oil creates the undesirable greasy/oily feel discussed above. Thus, the combined data from Tables 1-2, helped establish the relationship between alcohol level and repellency in a dose dependent manner and assisted in moving closer to an alcohol/oil ratio that would provide 100% repellency and the best feel on the skin.

TABLE 2

Results of repellency tests for selected formulations

Insect	*Aedes aegypti*						
Formula Name	Active, ingredients	1 hr	2 hr	3 hr	4 hr	6 hr	8 hr
Soybean 5	8% undecanone, 5% geraniol, 25% soybean oil, 62% isopropyl alcohol				*73.5% repellent		
Soybean 1	8% undecanone, 5% geraniol, 35% soybean oil, 52% isopropyl alcohol				*88.5% repellent		
Soybean 2	8% undecanone, 5% geraniol, 50% soybean oil, 37% isopropyl alcohol				*94.5% repellent		
Soybean 3	8% undecanone, 5% geraniol, 65% soybean oil, 22% isopropyl alcohol					*100% repellent	
Soybean 4	8% undecanone, 5% geraniol, 87% soybean oil					*100% repellent	

*200 mosquitoes in the cage, calculation of percentage repellency based on number of landings on treated arm versus untreated arm (see example 1 for calculation details); undecanone = 2-undecanone.

Example 4

The addition of specific emulsifiers prevented separation into multiple phases and improved the feel on the skin and the reduced transfer rates were maintained. However, the repellency of the formulations was reduced (Table 3). Also, the other negative characteristics (see Example 2, above) were still present. These data demonstrate the use of commercial grade denatured alcohol (ethanol) instead of isopropyl alcohol in the formulations. Based on dose response data in Table 1 and Table 2, it is believed that the reduced repellency of the formulation was a result of reducing the percentage soybean oil.

TABLE 3

Results of repellency tests for selected formulations

Insect	*Aedes aegypti*			
Formula Name	Active, ingredients	Control	4.0 hr	% repellency
Soybean 6	15% 2-undecanone, 5% polysorbate 20, 20% soybean oil, 55% denatured alcohol, 5% geraniol	205 landing/ min	29 landing/ min	*86%
Soybean 6	15% 2-undecanone, 5% polysorbate 20, 20% soybean oil, 55% denatured alcohol, 5% geraniol	240 landing/ min	139 landing/ min	*42%

*200 mosquitoes in the cage, calculation of percentage repellency based on number of landings on treated arm versus untreated arm (see example 1 for calculation details).

Example 5

Further experiments adjusting oil to alcohol ratio was needed to improve the desired repellency, maintain positive skin feel, to minimize accidental transfer from treated skin, to allow a stable monophasic system, and to eliminate issues associated with delivery of the repellent (issues as discussed in Example 2, above), see Table 4.

TABLE 4

Results of repellency tests for selected formulations

Insect	*Aedes aegypti*			
Formula Name	Active, ingredients	Control	4.0 hr	% repellency
Soybean 7	15% 2-undecanone, 5% polysorbate 20, 30% soybean oil, 40% denatured alcohol, 5% geraniol, 5% sorbitan oleate	372 landing/ min	23 landing/ min	94%
Soybean 7	15% 2-undecanone, 5% polysorbate 20, 30% soybean oil, 40% denatured alcohol, 5% geraniol, 5% sorbitan oleate	292 landing/ min	45 landing/ min	85%

*200 mosquitoes in the cage, calculation of percentage repellency based on number of landings on treated arm versus untreated arm (see example 1 for calculation details).

Example 6

By further reducing the percentage of alcohol and adjusting other components, a desired repellency of over 95% was achieved in combination with a stable monophasic system, which addressed each of the issues raised in Example 2 (above).

The formulations of the invention are advantageous in that they do not easily rub off the skin and do not result in irritation to the skin when applied. Additionally, the formulations of this invention have an excellent feel and better activity than other known insect repellent formulations (e.g., BioUD, as shown in "arm in cage" studies). Thus, for example, as reported in Witting-Bissinger et al. (J. Med. Entomol., 45:891-898 (2008)) a commercial composition of 7.75% 2-undecanone (BioUD) had a mosquito repellency of about 70% at 5 hours post application as compared to 99% at 5 hours for the formulations of the present invention. Notably, in Witting-Bissinger et al., the cages contained 50 mosquitoes as compared to 200 in the cages used to test the formulations of the present invention. Thus, the formulations provided better repellency under greater biting pressure.

Example 7 Additional Testing of the Formulations of the Invention

In this example, a formulation comprising 15% 2-undecanone, soybean oil, polysorbate 20, sorbitan oleate, oil of lemon eucalyptus and denatured alcohol was tested.

A colony of *Aedes aegypti* (L.) mosquitoes used in the bioassay was established from field-collected eggs from New Orleans, La., in 2003. At 6 to 8 month intervals, adults reared from field-collected eggs were added to the mosquito colony to sustain genetic diversity. Larvae were reared as described by Trexler et al. (2003). Mosquito colonies were maintained in the Dearstyne Entomology laboratory at 28° C., 75% relative humidity, and a photoperiod of 14:10 (L:D), including two twilight periods (60 min each). Adult females used in the experiments were provided 10% sucrose solution ad libitum and were never allowed to blood feed.

The testing procedures were as follows. The test area was the surface of the arm from the elbow to the wrist (hands were covered with non-allergenic gloves). Test areas were washed with soap and water then swabbed with 70% ethanol before application of each repellent. The ethanol was allowed to evaporate completely before repellent treatments. Control arms were cleansed in the same manner. For all studies on human skin, repellents were applied at a rate of 1 ml/600 cm2. For each test, mated nulliparous host-seeking (non-blood fed) female mosquitoes aged between 6 and 18 d were released into a square 27,000 cm^3 Plexiglas cage with a sleeved opening on one side for insertion of a subject's arm. The cage contained only the test mosquitoes during the testing period (no nectar or water source).

For the arm in cage test, the arm of the human subject (treated versus untreated control) was inserted into the cage for 1 minute. Tests were conducted at 25° C., 60-70% relative humidity and under florescence laboratory lighting. A fixed amount of the test compound (1 ml) was applied to one forearm by a researcher (the observer) who was wearing non-allergenic gloves on both hands. The observer applied the test compound using a P1000 Pipetteman with a sterile tip and then spread (and massaged into the skin using the pipette tip) from the elbow to the wrist and one arm of the subject (i.e., the treatment arm). Prior to the study, the pipette calibration was validated by transferring distilled

water to an analytical balance (+0.0001 grams) and was within the standard accuracy and precision of the manufacturer (<2% error). Nothing was applied to the other arm of the subject which was the control arm.

At different times after application of the formulation, the subject applied a non-allergic glove to the hand on the control and test arm and just afterward, inserted the arm into a cage (control arm first and then immediately the treatment arm); the cage contained up to 200 female mosquitoes. Each arm was inserted for 1 minute. The repellency was measured at multiple times post-treatment. Bioassays were conducted for each subject on the same cage of mosquitoes; different cages for different subjects. The time between bioassays was no shorter than 30 minutes and typically at least an hour. Insertion of the control versus treated arm did not cause a reduction in landing counts on the control arm at the different times tested. Between tests, the subject removed and disposed of the glove. For each time point, a new glove was used. The purpose of the glove was to prevent mosquito biting the hand, and no mosquito blood feeding on the hand was reported for any bioassay. The subject with a treated arm was encouraged to sit quietly and minimize physical activity.

Mosquitoes that touch the skin only are counted as landing; the mosquitoes do not have to stand on the skin with their legs to be considered a landing. When a landing was noticed where the mosquito is standing on the skin, the subject moved their arm in the cage in a rapid back and forth motion to prevent mosquito biting and blood feeding. No biting or blood feeding was noted in any of the bioassays. The observer counted the number of landings using a hand held numerical counter. The observer was positioned on the opposite side of the cage from the subject to best observe landings. The measurement of landings on the untreated arm (the control arm) preceded the measurements made on the treated arm. The mosquito density was adjusted to achieve 200 or more landings on the untreated arm within one minute.

Percentage repellency provided was calculated as follows: (control landings treatment landings/control landings)×100.

The formulation tested in each of Tables 5-8, below, comprised 15% undecanone, soybean oil, polysorbate 20, sorbitan oleate, oil of lemon eucalyptus and denatured alcohol.

TABLE 5

		At 4 hours		At 5 hours	
Subject	Control (land*/in)	Treatment (land/min)	% Repellency	Treatment (land/min)	% Repellency
1	320	19	94		
2	256	16	94		

*land = landings

TABLE 6

		At 4 hours		At 5 hours	
Subject	Control (land*/in)	Treatment (land/min)	% Repellency	Treatment (land/min)	% Repellency
1	312	1	99.7		
2	296	0	100		

*land = landings

TABLE 7

		At 4 hours		At 5 hours	
Subject	Control (land*/in)	Treatment (land/min)	% Repellency	Treatment (land/min)	% Repellency
1	156	2	98		
2	204	5	98		

*land = landings

TABLE 8

		At 4 hours		At 5 hours	
Subject	Control (land*/in)	Treatment (land/min)	% Repellency	Treatment (land/min)	% Repellency
1	216	3	99	2	99
2	468	3	99	4	99

*land = landings

The formulation of the invention was 99% effective for at least 5 hours in preventing mosquitoes even touching the subject's arm. In other trials with the formulation, the average repellency at 4 h was 95.3% (typical range 94 to 100%; Tables 5 to 7).

Example 8. The Effect of the New Formulations on the Foraging Behavior of Ticks and Mosquitoes

In this example, a formulation comprising 2-undecanone, soybean oil, polysorbate 20, sorbitan oleate, oil of lemon eucalyptus and denatured alcohol was tested.

The objective of these laboratory studies was to quantify foraging behavior of mosquitoes and ticks in response to human study participants that were treated with the formulations of the invention.

In one study, mosquito landings were recorded on the untreated versus repellent-treated arms of human subjects. Exposures to mosquitos were made at 30-minute intervals, with each exposure lasting for 1 minute for treated arms and for 20 seconds for untreated control arms (to limit the number of bites received). Control exposures served to verify biting avidity of mosquitoes during the study. Complete Protection Time was defined as the time from application of the test material to a subject's skin until the exposure in which the first landing with intent to bite occurred that was followed by another landing with intent to bite within the same or the next exposure interval.

In a second study, crossings of individual ticks on untreated and repellent-treated arms of human subjects were recorded. Fresh ticks were first selected for avidity by initially checking that the ticks would traverse a specified distance on each subject's untreated arm within a three-minute period. "Qualifying" ticks were those that walked upward on an untreated arm, toward the elbow. Any tick that qualified was then moved to that subject's treated arm in order to challenge the repellent. Complete Protection Time was defined as the time from application until the exposure in which there was a crossing confirmed by another crossing within 30 minutes (First Confirmed Crossing). The earlier of these crossings was considered the end of Complete Protection.

Each study included four subjects. In the case of the study of the foraging behavior of mosquitoes, the negative control was the untreated arm of one subject. In the case of the study

of the foraging behavior of ticks, the negative control was the untreated arm of each of the four subjects.

To minimize the chances of any potential substances (e.g., cosmetic products) on subject skin interfering with the test, each subject began by washing both arms with fragrance-free soap, rinsing with water, then washing both arms again with 70% ethyl alcohol in water, and finally rinsing with water. Arms were dried with plain paper towels prior to repellent applications. The topical repellent formulation of the invention was applied at a dosing rate of approximately of 1 mL per 600 cm2 of skin surface area. Accordingly, the total amount of material applied to each subject was determined by the surface area of the forearms of each subject.

Mosquito Testing

Mosquito testing was against adult female *Aedes aegypti* from laboratory-reared disease-free populations.

The formulation tested in this example comprised 2-undecanone, soybean oil, polysorbate 20, sorbitan oleate, oil of lemon eucalyptus and denatured alcohol (e.g., "the test material"). The test material was applied from the wrist to the elbow of each participant using a 1 mL syringe. Specifically, the repellent was distributed onto the skin in drops and lines, and then spread with two fingertips of one hand until complete and even coverage was observed. For each application, fresh gloves were used.

Approximately 100 laboratory-reared adult female mosquitoes were used to challenge the repellent in each exposure interval. Prior to testing, mosquitoes were supplied with feeding bottles of 10% sucrose in water. Feeding bottles were removed 12 hours prior to testing in order to increase their foraging avidity. Mosquitoes were held in 12-inch (~30 cm) cubic screened cages. During each exposure period, repellents were challenged in sequence in a single cage of mosquitoes. At 30-minute intervals beginning shortly after repellents were applied, each participant began exposures by inserting a forearm into a cage. For treated arms, exposures were for one minute, and for untreated control arms the exposure was for 20 seconds. Two observers counted mosquito landings during each exposure. Control exposures served to verify avidity in mosquitoes. A 20 second exposure of untreated arms to the mosquitoes was estimated to represent about 50% of the number that would be expected to land in a full minute exposure.

Repellent failure was scored independently for each subject, and defined as the time after application at which the first bite occurred that was followed by another bite within that or either of the next two intervals (time of First Confirmed Bite (FCB)).

Tick Testing

Tick tests used nymphal Deer Ticks, *Ixodes scapularis* from laboratory reared, disease-free populations and the formulation tested comprised 2-undecanone, soybean oil, polysorbate 20, sorbitan oleate, oil of lemon eucalyptus and denatured alcohol.

A single nymphal Deer Tick was used to challenge the repellent formulation of the invention during each exposure interval. Ticks were held in glass vials surrounded by water to contain them during the test, and fresh ticks were used for each challenge with a formulation of the invention. The formulation was challenged simultaneously in all subjects in 3 minute exposures commencing every 30 minutes beginning from the application of the test material.

Subjects manipulated ticks with small artist's paintbrushes, first placing them on an untreated arm to insure avid questing behavior in the absence of repellent, then placing them on the treated arm. In this way each subject served as their own negative control. Only avid ('qualified') ticks were used to challenge repellent formulations of the invention.

Crossings were measured by placing three dots on each subject's arms, 3-cm distant from one another, in a straight line (see, FIG. **1**). For a treated arm, repellent was present above the middle dot, and failure was defined as crossing 3-cm into the treated area, past the third dot. An exposure interval where a tick did continue past this third dot was scored as a crossing, and repellent was considered no longer effective at that point if that crossing was confirmed by another within that or either of the next two intervals (First Confirmed Crossing, or FCC). For controls, the same crossing criterion was used to insure tick avidity, but no treatment (i.e., no repellent was present).

Repellent failure was scored independently for each subject, and defined as the time after application in which the first crossing occurred that was followed by another crossing in the next two intervals (First Confirmed Crossing).

Results

Mosquitoes

Of the four test participants, two received confirming landing with intent to bite, for Complete Protection Time (CPT) of 3 and 5 hours, respectively. The remaining two subjects withdrew voluntarily. One of the subjects that withdrew voluntarily received a single landing with intent to bite at 2 hours and withdrew voluntarily at 4.5 hours and the other received a single landing with intent to bite at 6 hours and then withdrew. With two subjects experiencing repellent failures and two withdrawing before failure, only a conservative mean CPT value can be calculated. Mean±sd CPT was approximately 4.63±1.25 hours.

While exposures were initiated at 30-minute intervals, they were generally 5-10 min past the exact ½ hour intervals post application. Hence each of the hour values below understates protection duration by about 5-10 minutes. Accounting for minor exposure delays in each period increases the conservative mean of approximately 4.75 hours. Accepting that the subjects who withdrew could not have received confirming landing with intent to bite until at least 30 minutes after their withdrawal times, the minimum reasonable mean CPT calculation is probably about 5.0 hours.

Ticks

None of the four subjects experienced any crossings on the treated arms. All subjects withdrew after several hours of testing confirmed to evident prolonged complete protection. Two subject withdrew after 6.5 hours, and the other two withdrew after 7 hours. The mean±sd CPT against ticks was 6.75±0.25.

Against ticks, no subjects tested their treatment all the way to failure. Thus, longer protection times could have been recorded had all subjects done so. Nonetheless, the average protection time among the data gathered was reasonably long and showed no signs of failure.

In conclusion, the topical repellent formulation of the invention was effective against *Aedes aegypti* mosquitoes for about 5 hours, and against *Ixodes scapularis* ticks for at

least a few additional hours more than observed for the mosquitoes. We regard this duration of repellency against ticks to be strong.

Example 9. Animal Toxicity Tests

Any formulation for use as a repellant in addition to providing repellency at the same time has to be compatible to use on humans without adverse effects including not only skin but clothing, hair and accidental application to eyes and other sensitive surfaces on the body. It must also have no toxic effects and also have an acceptable relative to smell and feel on the skin and also relative to retention on treated surfaces with resistance to removal from typical rubbing that might occur to skin on different parts of the body. The formulations of the present invention were tested to show that they meet all of these criteria.

Test I. Skin Sensitization Study in Guinea Pigs

A formulation of the invention comprising 2-undecanone, soybean oil, polysorbate 20, sorbitan oleate, oil of lemon eucalyptus and denatured alcohol was tested for dermal sensitization potential utilizing a Buehler Technique Guinea Pig Sensitization Protocol. The test substance was evaluated for sensitization potential by applying 0.4 ml at a 100% concentration directly into Hilltop Chambers® and applying them to the clipped left shoulder of twenty albino guinea pigs in the following manner: The animals were held gently, and the chambers were applied as quickly as possible to the clipped left shoulder. The chambers were secured with Micropore tape and further secured with Kendall adhesive tape. Approximately six hours later, the tape and chambers were removed. Two additional induction doses were conducted following the same procedure, at weekly intervals.

Two weeks after the final application the animals received a topical primary challenge dose (6 hour contact) of the formulation at 100% concentration, on a naive site located on the right shoulder. Animals were scored for irritation at 24 and 48 hours after initiation of the primary challenge application.

Ten guinea pigs served as a naive control group, and remained untreated through the induction phase. Six naive control animals received only the primary challenge dose, at a 100% concentration. The four remaining guinea pigs were designated for a re-challenge, if necessary.

Following primary challenge with the formulation at 100% concentration, the incidence of grade 1 response or greater in the test group (0 of 20) was compared to that of the naive control group (0 of 6). The incidence and severity of these responses were not significantly greater than those produced by the naive control group indicating that sensitization had not been induced.

Test II. Acute Inhalation Toxicity Study in Rats

A formulation of the invention comprising 2-undecanone, soybean oil, polysorbate 20, sorbitan oleate, oil of lemon eucalyptus and denatured alcohol was tested for acute inhalation toxicity in accordance with OPPTS Guideline 870.1300 and OECD Guideline 403 at a targeted level of 2.0 mg/L of air by exposing five male and five female rats for a four hour period. The actual gravimetric concentration of the study was 2.33 mg/L of air. No mortality was observed during the 14 day observation period. The inhalation LC50 of the formulation was found to be greater than 2.33 mg/L of air for a 4 hour period. In accordance with the OPPTS Guidelines, formulation of the invention would be classified as EPA Toxicity Category IV for acute inhalation toxicity.

Test III. Acute Dermal Toxicity Study in Rabbits

A formulation of the invention comprising 2-undecanone, soybean oil, polysorbate 20, sorbitan oleate, oil of lemon eucalyptus and denatured alcohol was tested for acute dermal toxicity according to OPPTS, OECD Guidelines. The formulation, an amber liquid, was administered by dermal application at a dose of 5.0 g/kg body weight to five male and five female rabbits. No mortality occurred during the 14 day observation period. The acute dermal LD50 was found to be greater than 5.0 g/kg body weight. The formulation of the invention would be classified as Toxicity Category IV for dermal toxicity

Test IV. Acute Oral Toxicity Study in Rats

A formulation of the invention comprising 2-undecanone, soybean oil, polysorbate 20, sorbitan oleate, oil of lemon eucalyptus and denatured alcohol was tested for acute oral toxicity in accordance with OPPTS 870.1100 and OECD 425 Guidelines. The formulation, an amber liquid, was administered by oral gavage at a 5000 mg/kg body weight limit dose level, according to the Agency's Up and Down Procedure to female rats. The acute oral LD50 was determined to be greater than 5000 mg/kg body weight. Therefore, in accordance with OPPTS Guidelines, the formulation of the invention meets the requirements for EPA Toxicity Category IV for acute oral toxicity.

Test V. Acute Dermal Irritation Study in Rabbits

A formulation of the invention comprising 2-undecanone, soybean oil, polysorbate 20, sorbitan oleate, oil of lemon eucalyptus and denatured alcohol was tested for primary dermal irritation in accordance with OPPTS and OECD Guidelines. The formulation, an amber liquid, was applied at a dose of 0.5 ml to an unabraded site on a clipped area of each of three albino rabbits. The application sites were graded for indication of skin reactions at 0.5, 24, 48, 72, 168 and 336 hours after test substance removal. There were mild skin irritation reactions in all of the test subjects which cleared by the 336 hour observation. The maximum skin irritation score was 3.0 at the 48 hour observation. The primary skin irritation index was calculated to be 2.58. In accordance with the OPPTS Guidelines, the formulation of the invention would be classified as EPA Toxicity Category IV for dermal effects.

Test VI. Acute Eye Irritation Study in Rabbits

A formulation of the invention comprising 2-undecanone, soybean oil, polysorbate 20, sorbitan oleate, oil of lemon eucalyptus and denatured alcohol was tested for eye irritation in accordance with OPPTS and OECD Guidelines. The formulation, an amber liquid, was administered into one eye of each of three albino rabbits. The eyes were observed and scored at 1, 24, 48, 72 and 168 hours. There was moderate eye irritation observed in all of the three test subjects which cleared by the 168 hour observation. The maximum group mean score was 14.67/110.0 at the 24 hour observation. In accordance with the OPPTS Guidelines, the formulation of the invention would be classified as Toxicity Category III.

Example 10. Human Repeat Insult Patch Tests for Skin Irritation and Skin Sensitization Evaluation

To determine the irritation and sensitization (contact allergy) potential of a test material after repeated application to the skin of human subjects three studies were conducted using the formulations of the invention comprising 2-undecanone, soybean oil, polysorbate 20, sorbitan oleate, oil of lemon eucalyptus and denatured alcohol (e.g., test materials).

Procedures

Test materials to be tested under occlusive conditions were placed on an 8-millimeter aluminum Finn Chamber® (Epitest Ltd. Oy, Tuusula, Finland) supported on Scanpor® Tape (Norgesplaster A/S, Kristiansand, Norway) or an 8 mm filter paper coated aluminum Finn Chamber® AQUA supported on a thin flexible transparent polyurethane rectangular film coated on one side with a medical grade acrylic adhesive, consistent with adhesive used in state-of the-art hypoallergenic surgical tapes or a 7 mm IQ-ULTRA® closed cell system which is made of additive-free polyethylene plastic foam with a filter paper incorporated (It is supplied in units of 10 chambers on a hypoallergenic nonwoven adhesive tape; the width of the tape is 52 mm and the length is 118 mm) or other equivalents. Materials to be tested under semi-occlusive conditions were placed on a test strip with a Rayon/Polypropylene pad or on a 7.5 mm filter paper disc affixed to a strip of hypoallergenic tape (Johnson & Johnson 1 inch First Aid Cloth Tape).

Test materials to be tested in an open patch were applied and rubbed directly onto the back of the subject. Approximately 0.02-0.05 mL (in case of liquids) and/or 0.02-0.05 gm (in case of solids) of the test material was used for the studies. Liquid test material was dispensed on a 7.5 mm paper disk, which fit in the Finn Chamber.

Subjects were requested to bathe or wash as usual before arrival at the facility. Patches containing the test material were then affixed directly to the skin of the intrascapular regions of the back, to the right or left of the midline and subjects were dismissed with instructions not to wet or expose the test area to direct sunlight. Patches remained in place for 48 hours after the first application. Subjects were instructed not to remove the patches prior to their 48 hour scheduled visit. Thereafter, subjects were instructed to remove patches 24 hours after application for the remainder of the study.

This procedure was repeated until a series of nine (9) consecutive, 24-hour exposures had been made three (3) times a week for three (3) consecutive weeks. Prior to each reapplication, the test sites were evaluated by trained laboratory personnel. Following a 10-14 day rest period a retest/challenge dose was applied once to a previously unexposed test site.

Test sites were evaluated by trained laboratory personnel 48 and 96 hours after application.

In the event of an adverse reaction, the area of erythema and edema were measured. Edema was estimated by the evaluation of the skin with respect to the contour of the unaffected normal skin. Subjects were instructed to report any delayed reactions that might occur after the final reading.

Scoring scale and definition of symbols shown below are based on the scoring scheme according to the International Contact Dermatitis Research Group scoring scale [Rietschel, R. L., Fowler, J. F., Ed., Fisher's Contact Dermatitis (fourth ed.). Baltimore, Williams & Wilkins, 1995] listed below:

0 no reaction (negative)

1 erythema throughout at least ¼ of patch area

2 erythema and induration throughout at least ¾ of patch area

3 erythema, induration and vesicles

4 erythema, induration and bullae

D Site discontinued

Dc Subject discontinued voluntarily

DcI Subject discontinued per Investigator

Study I

Formulation: 2-undecanone, soybean oil, polysorbate 20, sorbitan oleate, oil of lemon eucalyptus and denatured alcohol.

Population Demographics

Number of subjects enrolled	66
Number of subjects completing study	59
Age Range	19-65
Sex	
Male	4
Female	55
Fitzpatrick Skin Type*	
1 - always burn, does not tan	0
2 - burn easily, tan slightly	22
3 - burn moderately, tan progressively	8
4 - burn a little, always tan	17
5 - rarely burn, tan intensely	12
6 - never burn, tan very intensely	0

*Agache P., Hubert P.. *Measuring the skin.* Springer-Verlag Berlin Heidelberg, 2004, (p. 473, table 48.1)

Results: No adverse reactions of any kind were reported during the course of this study. There were thirty-five (35) subjects with a Grade 1 reaction and four (4) subjects with a Grade 1 delayed reaction to the positive control (2.0% Sodium Lauryl Sulfate Solution). No subjects showed any signs of reaction to the negative control (DI Water).

Study II

Formulation: 2-undecanone, soybean oil, polysorbate 20, sorbitan oleate, oil of lemon eucalyptus and denatured alcohol.

Population Demographics

Number of subjects enrolled	55
Number of subjects completing study	55
Age Range	18-64
Sex	
Male	9
Female	46
Fitzpatrick Skin Type*	
1 - always burn, does not tan	0
2 - burn easily, tan slightly	0
3 - bum moderately, tan	55
4 - bum a little, always tan	0
5 - rarely burn, tan intensely	0
6 - never burn, tan very intensely	0

*Agache P., Hubert P.. *Measuring the skin.* Springer-Verlag Berlin Heidelberg, 2004, (p. 473, table 48.1)

Results: No adverse reactions of any kind were reported during the course of this study. There were Ten (10) subjects with a Grade 1 reaction to the positive control (2.0% Sodium Lauryl Sulfate Solution). No subjects showed any signs of reaction to the negative control (DI Water).

Study III

Formulation: 2-undecanone, soybean oil, polysorbate 20, sorbitan oleate, oil of lemon eucalyptus and denatured alcohol.

Population Demographics

Number of subjects enrolled	55
Number of subjects completing study	54
Age Range	19-63
Sex	
Male	5
Female	49
Fitzpatrick Skin Type*	
1 - always burn, does not tan	0
2 - burn easily, tan slightly	0
3 - burn moderately, tan progressively	54
4 - burn a little, always tan	0
5 - rarely burn, tan intensely	0
6 - never burn, tan very intensely	0

*Agache P., Hubert P.. *Measuring the skin.* Springer-Verlag Berlin Heidelberg, 2004, (p. 473, table 48.1)

Results. No adverse reactions of any kind were reported during the course of this study. Two subjects showed Grade 1 and two subjects showed Grade 2 reaction to the positive control (2.0% Sodium Lauryl Sulfate Solution). No subjects showed any signs of reaction to the negative control (DI Water).

The foregoing is illustrative of the present invention, and is not to be construed as limiting thereof. The invention is defined by the following claims, with equivalents of the claims to be included therein.

That which is claimed is:

1. A monophasic formulation comprising:

2-undecanone in an amount of about 5% to about 50% by volume in the formulation;

at least one hydrophobic base in an amount of about 5% to about 65% by volume in the formulation;

at least one alcohol in an amount of about 5% to about 50% by volume in the formulation; and

at least one emulsifier in an amount of about 5% to about 15% by volume in the formulation.

2. The monophasic formulation of claim **1**, further comprising at least one of oil of lemon eucalyptus (OLE), p-menthane-3,8-diol, and/or geraniol.

3. The monophasic formulation of claim **1**, wherein the at least one hydrophobic base is a branched hydrocarbon, an unbranched hydrocarbon, a hydrophobic polymer, a fatty acid, a fatty acid ester, a monoglyceride, a diglyceride, and/or a triglyceride.

4. The monophasic formulation of claim **1**, wherein the at least one hydrophobic base is a plant-based oil, optionally avocado oil, borage oil, canola (rapeseed) oil, castor oil, chamomile oil, coconut oil, corn oil, cottonseed oil, rapeseed oil, evening primrose oil, safflower oil, sunflower oil, soybean oil, sweet almond, palm oil, palm kernel oil, *Arctium lappa* seed oil, sesame oil, borgo officialis seed oil, *Brassica campestris* oleifera oil, brevoortia oil, bubulum oil, cistus ladaniferus oil, *Elaeis guineensis* oil, almond oil, pine oil, olive oil, peanut oil, flax seed oil, wheat germ oil, hemp oil, jojoba oil, linseed oil, macadamia oil, neem oil, argan oil, grape seed oil, thistle oil, and/or algal oil and/or any combination thereof.

5. The monophasic formulation of claim **4**, wherein the plant-based oil is soybean oil, cottonseed oil and/or coconut oil.

6. The monophasic formulation of claim **1**, wherein the at least one alcohol is ethanol, isopropyl alcohol, t-butyl alcohol, propyl alcohol, and/or benzyl alcohol.

7. The monophasic formulation of claim **1**, wherein the at least one alcohol is ethanol, isopropyl alcohol and/or denatured alcohol.

8. The monophasic formulation of claim **1**, wherein the at least one emulsifier is polysorbate 20, sorbitan oleate, potassium sorbate, and/or sodium benzoate.

9. The monophasic formulation of claim **1**, wherein the formulation comprises an equal amount of the at least one alcohol to the at least one hydrophobic base.

10. The monophasic formulation of claim **1**, wherein the formulation comprises the at least one hydrophobic base in an amount that is about two times greater than the amount of the at least one alcohol.

11. The monophasic formulation of claim **1**, wherein the formulation comprises the at least one alcohol in an amount that is about 3% to about 10% (vol./vol.) greater than the amount of the at least one hydrophobic base.

12. The monophasic formulation of claim **1**, wherein the formulation comprises oil of lemon eucalyptus (OLE) in an amount of about 5% to about 15% by volume in the formulation.

13. A product infused with the monophasic formulation of claim **1**, optionally wherein the product is a textile, a plastic, latex, rubber, cellulose, a wick, a candle, paint, a cosmetic, a lotion, a shampoo, a conditioner, and/or a mousse.

14. The product of claim **13**, wherein the textile is clothing, bedding, linens, towels, curtains, draperies, tents, and/or netting.

15. A method of repelling an insect pest or a tick, comprising:

applying the monophasic formulation of claim **1** to a subject or a substrate in an amount effective to repel the insect or tick.

16. The method of claim **15**, wherein the insect is a mosquito, sand fly, filth fly, chigger, flea, black fly, deer fly, horse fly, lice, roach, ant, and/or fire ant.

17. The method of claim **15**, wherein about 90% to about 100% of the insects and/or ticks are repelled, optionally about 95% to about 100% or about 98% to about 100% of the insects and/or ticks are repelled.

18. The method of claim **15**, wherein the insects and/or ticks are repelled for up to about 7 hours after application.

19. The method of claim **15**, wherein the subject is a human.

20. The method of claim **15**, wherein the substrate is fabric, cotton products, clothing, wrist bands, bedding, linens, towels, curtains, draperies, wood products, furniture, carpet, floors, walls, baseboards, netting, paper, water, trash containers, dumps, tents, paint, plants, and/or soil.

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