Spatial Repellency and the Field Evaluation of a Push-Pull Strategy for the Control of Malaria Vectors in Northern Belize, Central America

by

Joseph M. Wagman

Dissertation submitted to the Faculty of the
Emerging Infectious Diseases Graduate Program
Uniformed Services University of the Health Sciences
In partial fulfillment of the requirements for the degree of
Doctor of Philosophy 2014

UNIFORMED SERVICES UNIVERSITY, SCHOOL OF MEDICINE GRADUATE PROGRAMS
Graduate Education Office (A 1045), 4301 Jones Bridge Road, Bethesda, MD 20814

APPROVAL OF THE DOCTORAL DISSERTATION IN THE EMERGING INFECTIOUS DISEASES GRADUATE PROGRAM

Title of Dissertation: "Spatial Repellency and the Field Evaluation of a Push-Pull Strategy for the Control of Malaria Vectors in Northern Belize, Central America"

Name of Candidate: Joseph Wagman
Doctor of Philosophy Degree
September 18, 2014

DISSERTATION AND ABSTRACT APPROVED:

DATE:

Stephen J. Davies, Ph.D. — 9/18/14
DEPARTMENT OF MICROBIOLOGY AND IMMUNOLOGY
Committee Chairperson

John P. Grieco, Ph.D — 9/18/14
DEPARTMENT OF PREVENTIVE MEDICINE AND BIOMETRICS
Dissertation Advisor

D. Scott Merrell, PhD. — 9/18/14
DEPARTMENT OF MICROBIOLOGY AND IMMUNOLOGY
Committee Member

Phillip E. Coyne. Ph.D. — 18 Sept 14
DEPARTMENT OF PREVENTIVE MEDICINE AND BIOMETRICS
Committee Member

Cara H. Olsen. DrPH — 9/18/14
DEPARTMENT OF PREVENTIVE MEDICINE AND BIOMETRICS
Committee Member

Nicole L. Achee. Ph.D. — 9/18/14
DEPARTMENT OF BIOLOGICAL SCIENCES, ECK INSTITUTE, UNIVERSITY OF NOTRE DAME
Committee Member

ACKNOWLEDGMENTS

Thanks to John and Nicole for mentoring, encouraging and guiding me through the lab and the field, and for trusting me to swim when I could have easily sunk.

Thanks to the members of my qualifying and dissertation committees for continual guidance and support: John P. Grieco, Ph.D.; Nicole L. Achee, Ph.D.; Stephen Davies, Ph.D.; Phillip Coyne, MD; Cara Olsen, Dr.P.H.; D. Scott Merrell, Ph.D.; Gerald Quinnan, Jr, MD; and Christopher Broder, Ph.D. Thanks to Angela Caranci Wright for joining the lab, choosing to bring non-mosquito diversity to our project lineup, and being a great friend and colleague. Special thanks to the offices of GEO and PMB, particularly Eleanor Metcalf, Ph.D., Maria Smith and Hang Tran, for invaluable support with all things finance and travel-related.

Thanks to USUHS and to the Bill and Melinda Gates foundation for generously funding our projects. Thanks to Maude Meier, Ph.D., of S.C. Johnson and Son, Inc. for graciously supplying the test repellent and guidance on its field application.

Thanks to the proud nation of Belize and its limitless hospitality, particularly the community of Orange Walk, for welcoming my wife and me with open arms and for tacos and garnachas, three for a dollar. Much appreciation to Mr. Albert Burns for the generous use of his land and advice about avoiding the jaguar, and to Lisbet Escalante, Rosana Briceño and Johnny Briceño for your invaluable friendship.

Thanks to the Ministry of Health, Belize for protocol approval, support, and encouragement as well the opportunity to serve the Belizean community through many and varied collaborations with the national Vector Control Program. Special acknowledgement to Dr. Aisha Andrewin, Kim Bautista, 'Mac' MacGreggor, Michael

Pitts, and Dr. Jorge Polanco from the Ministry of Health and to Ms. Miriam Serrut of the Belize Pesticides Control Board.

Thanks to Russel King, Ireneo Briceño, and Chris Martinez for making up the core of 'the team' that consistently saw that everything got done. Thanks also to the rest of the *mosquiteros* and their families for the hours you spent with us collecting (among other things) mosquitoes: Louis Balam, Ian Briceño, Ireno Briceño, Jr, Susana Castillo, Angela Caranci-Wright, Selvin García, Abner Gómez, Russell King, Jr, Adrian Tillett, German Tillett, José Tillett and Marcelo Urbina.

Thanks to my parents, Mike and Jeanne; my sisters, Lisa and Maria; and the rest of my family, who have ALWAYS supported and encouraged me to do what makes me happy, even when it has taken me far away from home.

Finally, the sincerest thanks to my loving wife, Susana, whose unending support (physical, emotional, and financial) and sense of adventure helped fuel this experience from start to finish. *Tu eres más que mi corazón; eres también mi inspiración.*

"You shall bite each one of them in turn. Bite the first one seated there, and then keep biting them until you have finished biting all of them. This is the part you must play: to suck the blood of the men on the road."

– Instructions given to the mosquito spy, Xa'n, by the Mayan hero twins Hunahpú and Xbalanqué during their journey into the underworld. From *Popol Wuj*, the Book of the People: Part II, Chapter 8 Verse 2

COPYRIGHT STATEMENT

The author hereby certifies that the use of any copyrighted material in the dissertation manuscript entitled: "Spatial Repellency and the Field Evaluation of a Push-Pull Strategy for the Control of Malaria Vectors in Northern Belize, Central America" is appropriately acknowledged and, beyond brief excerpts, is with the permission of the copyright owner.

Joseph M. Wagman

ABSTRACT

Spatial Repellency and the Field Evaluation of a Push-Pull Strategy for the Control of Malaria Vectors in Northern Belize, Central America

Joseph M. Wagman, Doctor of Philosophy, 2014

Thesis directed by: John P. Grieco, Ph.D., Associate Professor
Department of Preventive Medicine and Biometrics

Mosquito-borne diseases represent one of the most significant threats to human health worldwide, with hundreds of millions of infections resulting in extraordinary morbidity and mortality every year. Although vector control interventions are among the most effective known ways to prevent the transmission of mosquito-borne diseases, new strategies and paradigms are desperately needed to complement currently available tools. One novel approach to vector control currently being considered is a combined push-pull strategy, which uses spatial repellents and baited traps simultaneously in order to reduce human-vector interactions and to facilitate the capture and removal of vectors from the local environment. Here, a series of interdisciplinary studies was conducted to evaluate the potential for push-pull strategies to control mosquito vectors of human disease and to better define the physiological basis of spatial repellency.

First, we used a field-based experimental hut methodology to evaluate a prototype push-pull intervention against two natural malaria vector populations, *Anopheles albimanus* and *An. vestitipennis*, in northern Belize, Central America. Results show that the combined strategy can decrease human-vector interactions both indoors, by reducing mosquito entry into human-occupied huts (both *An. vestitipennis* and *An. albimanus*), and outdoors, by increasing mosquito trap efficacy (*An. vestitipennis* only). While these

results provide clear evidence that the combined strategy has potential to limit disease transmission in and around homes, the variation in effect seen on different target vectors highlights the need to characterize the underlying behavioral ecology of mosquitoes in order to drive intervention optimization in varied transmission settings.

Secondly, we used an *in vitro* behavioral bioassay to investigate the plasticity, heritability and physiological basis of spatial repellency behaviors in the global arbovirus vector, *Aedes aegypti*. While results indicate that spatial repellency is a relatively plastic behavior that likely results from a combination of factors, some heritable and others non-heritable, we were able demonstrate that transfluthrin repellent insensitivity is a recessive trait linked to reduced insecticide susceptibility in selectively bred mosquito populations. This provides novel evidence that the neurotoxic irritation of mosquitoes by sub-lethal doses of airborne insecticide is one of the primary mechanisms by which some chemicals can elicit spatial repellency behaviors in *Ae. aegypti*, and raises some important questions about how the long term use of certain spatial repellents, namely volatile pyrethroids, might impact vector populations over time.

Collectively, the work presented here provides a valuable proof-of-principle for push-pull mosquito control strategies and supports further investment into the optimization and validation of the approach for disease vector control while highlighting the need to identify new insect behavior modifying compounds with novel, non-toxic mechanisms of action.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1: General Introduction[1]

THE GLOBAL BURDEN OF VECTOR-BORNE DISEASES

Generally defined as any infectious disease whose etiologic agent is transmitted from one individual to another by an arthropod (46), vector-borne diseases represent one of the most significant threats to human health worldwide (Fig. 1). Any cursory listing of 'important' infectious diseases is likely to include many that are vector-borne: malaria, dengue fever, leishmaniasis, yellow fever, Lyme disease, West Nile virus, Rift Valley fever, plague, and chikungunya are all currently listed as leading health and research topics by the World Health Organization and/or the US Centers for Disease Control and Prevention (28; 171). It is not without good reason that vector-borne diseases receive so much publicity and attention given that, as a group, they account for around 17% of the entire global burden of infectious disease (a staggering 60 million disability adjusted life years lost every year) (103; 149; 160).

Although vector-borne pathogens are present in virtually every country and may currently infect up to several billion people worldwide (103; 149; 159), it is important to emphasize that these diseases disproportionally affect those living in impoverished and marginalized communities, mostly in the tropics (73; 103; 149; 159; 160). Indeed, vector-borne infections contribute much to the vicious cycle of poverty, which tends to

---

[1] Portions of chapter 1 have been adapted from:
Wagman J, Grieco J, Bautista K, Polanco J, Briceño I, King R and Achee N. 2014. A Comparison of Two Commercially Available Adult Mosquito Traps for the Capture of Malaria Vectors in Northern Belize, Central America. *J Am Mosq Control Assoc* 30:000 (In Press)
and
Wagman J, Grieco J, Bautista K, Polanco J, Briceño I, King R and Achee N. In review. The Field Evaluation of a Push-Pull Strategy to Control Malaria Vectors in Northern Belize, Central America. *PLoS ONE* submission # PONE-D-14-18319

simultaneously increase exposure to disease and create underlying conditions that exacerbate poor health outcomes, further limiting opportunities for the general development, personal empowerment and increased productivity needed to break the grip of poverty (26; 56). For example, it is estimated that every year the febrile illness malaria (which is caused by parasitic protozoa of the genus *Plasmodium* that are transmitted by the bite of anopheline mosquitoes) causes more than 500 million clinical infections (139), contributes to around one million deaths (169), and costs tens of billions of US dollars in lost productivity (56). These cases are almost entirely in underdeveloped regions of Sub-Saharan Africa, Southeast Asia and Latin America (139; 169).

Though the specter of malaria in the developing world is certainly prolonged and somewhat intractable, it is not the only mosquito-borne disease with the potential for widespread or profound effects on public health and human development. Dengue virus, spread primarily by the bite of *Aedes aegypti* (L.), is the most common arthropod-borne virus in man and causes between 50-100 million infections every year (17). Dengue virus infections present with a range of clinical conditions from asymptomatic viremia and classical dengue fever ('breakbone' fever) to the much more serious conditions of dengue hemorrhagic fever and dengue shock syndrome. At least 2.5 billion people are living in dengue endemic areas (17), though that number is likely to increase as dengue virus continues to expand in range (96; 153). Large outbreaks of this disease are estimated cost hundreds of millions of dollars in direct costs and lost productivity (142). Other emerging and re-merging mosquito-borne diseases of public health importance, both in and outside of the tropics, include West Nile fever, Chikungunya fever, Rift Valley fever and several encephalitis causing alpha viruses, to name only a few (46; 157).

All things considered, it is safe to say that a significant proportion of the world's burden of disease is transmitted through the bite of a mosquito (149; 160) and that mosquito-borne diseases (and their prevention) will remain at the forefront of global public health research and policy priorities for the foreseeable future (157; 167).

A Case for Vector Control

Vector control interventions are among the most effective methods of preventing mosquito-borne infections and have been shown to dramatically reduce disease rates when properly implemented (39; 47; 149; 168). A vector control intervention can be broadly defined as any action that reduces contact between vectors and humans, and the principle is deceptively simple: the prevention of arthropod bites results in the prevention of disease (through reduced pathogen transmission). For malaria and dengue fever, the two most globally significant mosquito-borne diseases today, the importance of vector control is easily illustrated.

The first malaria vector control campaigns, including those that helped eliminate the disease from large portions of North America and Europe in the early 20th century, focused on environmental modification to reduce mosquito breeding sites and basic house improvements like screened windows and closed eves (36; 162). The former strategy reduces disease transmission by decreasing overall vector population densities, while the later functions by directly limiting contact between vectors and humans. Although the impact of these interventions on disease incidence was clear in many settings, success was geographically, politically and economically limited to countries that were already on the margins of malaria endemicity and large scale multi-national elimination and eradication were not seriously discussed (80; 162; 168). This perspective

changed in the 1940's with the discovery of the residual and insecticidal properties of dichlorodiphenyl-trichloroethane (DDT) and the development of indoor residual spraying (IRS) as a strategy to combat disease (36; 47; 80).

IRS is the application of long-lasting insecticides to the inside walls (and sometimes roofs and eves) of a house in order to control disease vectors that would normally enter the house to feed on a human host (122; 161). As early as 1943, it was evident that IRS with DDT produced significant reductions in indoor malaria transmission and house spraying programs in the United States, Europe, South America and India produced rapid and encouraging results (126).

In part because of this incredible success of IRS, large scale elimination of the disease seemed feasible and the Global Malaria Eradication Program was launched by the World Health Assembly in 1955 (36; 80; 162; 168). Although the Global Program did lead to the elimination of malaria from Europe, Russia, and several countries in the Caribbean and Asia (161), ultimate success fell short of the goal of eradication and the program was abandoned in the 1970's in favor of long-term malaria control activities that were to be integrated into every endemic countries' national primary health care system (57; 80). The aftermath of this shift in strategies was relatively disastrous as vector control programs languished and malaria incidences roared back to pre-eradication program levels in many areas, particularly in tropical America and South and Southeast Asia, while in large portions of Africa (where the eradication program was never fully implemented to begin with) the malaria situation also continued to deteriorate (36; 123; 126; 156).

More recently, another round of coordinated efforts aimed at dramatically reducing the global burden of malaria has been made by, among others, the World Health Organization's Roll Back Malaria initiative, the US President's Malaria Initiative, and the Bill and Melinda Gates Foundation (47; 52; 98). These recent initiatives have enjoyed much success in reducing malaria burden locally and regionally, and have once again engendered a cautious optimism about future prospects of further elimination and eradication (15; 67; 169). Importantly, vector control has been an integral part of this recent success, mostly through IRS and the distribution of long lasting insecticide treated bed nets (LLINs), and is recognized as a cornerstone of malaria control efforts moving forward (47; 80; 169).

A historical overview of dengue vector control is not nearly as long or storied as that of malaria vector control, but it does offer some interesting similarities. In much of the Americas during the 1950's and 1960's, widespread *Ae. aegypti* eradication campaigns organized by the Pan American Health Organization were successful in interrupting dengue transmission across much of the continent (109; 165). But, the vector was never truly eliminated and intense surveillance and control programs were abandoned for many of the same technical and systemic reasons that the Global Malaria Elimination Program was abandoned. Accordingly, there were widespread resurgences of both the mosquito and the virus (109; 165). Nonetheless, given the lack of any acceptable vaccine, specific treatment or chemoprophylaxis options for dengue virus infection, it is often correctly emphasized that vector control is really the only public health tool available to help prevent the disease (51; 109), a paradigm that also holds true

for most other arbovirus infections (with one notable exception being yellow fever, for which a efficacious vaccine is widely available).

THE NEED FOR NEW TOOLS

Despite numerous historical and recent successes, and the current re-emphasis on the effectiveness and importance of targeted vector control, the tools at our disposal today are inadequate in many disease transmission settings (59; 85; 167). In contemporary malaria prevention programs the most widely recommended vector control strategies are IRS and the use of LLINs. Though generally these tools are still of tremendous public health value, in many locations their efficacy to control disease is either declining or of limited impact for a variety of reasons, among which are the emergence of insecticide resistance and vector behaviors (e.g. daytime or outdoor-biting) not optimally impacted by indoor nets or sprays (13; 14; 47; 63).

The standard approach to dengue control, advocated by WHO as part of the broader integrated vector management (IVM) philosophy, highlights surveillance and monitoring via the establishment of larval indices to prioritize risk areas and focus public outreach. These activities are usually coupled with source reduction and larvaciding interventions, while spraying with adulticides, typically from truck-mounted ultra-low volume sprayers or hand-held thermal foggers, can be carried out seasonally and/or as part of an outbreak response (165; 168). Unfortunately, the ultimate value of these interventions at preventing disease is not well validated or widely demonstrated (18; 50; 75), and there are concerns that emerging insecticide resistance in *Aedes* populations could further complicate control efforts (16; 30; 32).

Given the degree to which global vector control regimens rely on the use of chemicals to kill adult mosquitoes (88; 102), it is worrisome that there are only 15 active ingredients from four chemical classes currently recommended by WHO for use as public health insecticides and no new compounds are expected to become available for use in the coming years (Table 1) (8; 70; 163). Probably the most widely used insecticide class, the pyrethroids, are the only chemicals recommended for use on insecticide treated bed nets (164), and represent a full 50% (6 out of 12) of those active ingredients recommended for IRS (166). Not surprisingly, mosquito resistance to pyrethroids is spreading rapidly worldwide (70; 102), although resistance to each of the other three insecticide classes is also frequently and widely reported (77).

Additional challenges that can complicate vector control operations include widespread public concerns over the unintended consequences of pesticide over-exposure (126) and the tacit recognition that, despite the deceptively simple 'prevention of bites = prevention of disease' paradigm, the ecology and epidemiology of vector-borne disease is actually quite complex and there will never be a one-size-fits all vector control strategy to be universally successful across all disease transmission settings (168). When these limitations are considered against the backdrop of growing concern over the expanding impact and global distribution of arboviruses and renewed calls for the elimination and eradication of malaria, it becomes clear that new tools and innovative strategies are desperately needed in order to achieve maximum, sustained success from vector control interventions (47; 85; 148; 149; 159; 167).

A Novel Approach: Push-Pull Strategies and the Exploitation of Spatial Repellency

Push-pull strategies for vector control are one innovative approach under development that could help address this need for new tools and novel paradigms. The basic principle of a push-pull approach is to integrate the use of spatial repellents (a 'push' stimulus, which deters host-seeking mosquitoes from entering treated areas) and baited traps (a 'pull' stimulus, which attracts the mosquitoes to traps using chemical lures) to reduce human-vector interactions and to facilitate the capture and removal of vectors from the peridomestic environment in order to decrease the probability of pathogen transmission (38; 82; 90; 111).

The push-pull strategy

Formally named and introduced by Pyke and others in 1987 as a strategy to control pests of cotton crops in Australia (114), the push-pull strategy of pest control is defined by Cook and others (2007) as the behavioral manipulation of insects

"...via the integration of stimuli that act to make the protected resource unattractive or unsuitable to the pests (push) while luring them toward an attractive source (pull) from where the pests are subsequently removed (38). "

Though the push-pull strategy flows from a relatively intuitive and straightforward concept (Fig. 2A), selecting the best components for an intervention and understanding how they will interact with one another to affect arthropod behaviors can be quite complex. Nonetheless, the approach has been successfully developed and utilized in the agricultural sector to control a variety of crop-damaging insects like

lepidopteran pests of corn and sorghum, *Helicoverpa* spp. (pests of cotton), *Sitona lineatus* (a pest of legumes), the Colorado potato beetle (*Leptinotarsa decemlineata*), and the onion maggot *Delia antiqua*, among others (38; 114; 117). Given these successes, a natural question for public health scientists and vector biologists to consider is whether or not the push-pull approach can be adapted for the improved control of human disease vectors (Fig. 2B).

While this expanded use of push-pull strategies for public health applications is still in the proof-of-concept phase, preliminary developments appear promising. In Africa, Kitau and others (2010) have shown in a semi-field environment that the combined use of personal repellents (topically applied) and mosquito traps could reduce the biting rates of laboratory reared *Anopheles gambiae* more than the use of traps alone (82). Iwashita and others (2014), working in an area of the Lake Victoria region of western Kenya with high LLIN coverage, collected mosquitoes from inside houses and found that human feeding rates decreased for *An. arabiensis* in the presence of both pyrethroid-treated LLINs and increasing numbers of nearby cattle (but not goats and sheep), suggesting a mechanism in which mosquitoes might be 'pushed' away from human contact by bed nets and 'pulled' towards cattle for an alternative blood meal (78). In addition, others continue to demonstrate progress towards defining the parameters of a push-pull intervention for the control *Aedes aegypti* (7; 131; 132; 146), including studies demonstrating local interest and community buy-in into the concept in both Latin America and South East Asia (111). However, if push-pull strategies for vector control are ever to mature into viable public health policy, proof-of-concept and optimization

studies must validate the approach in varied and diverse disease transmission settings. Furthermore, a better understanding of how repellents work is needed.

Spatial repellency behaviors in mosquitoes

Instead of relying on the direct killing of adult mosquitoes, push-pull strategies for vector control aim to exploit the spatial repellency (SR) action of public health chemicals, a phenomenon that is well documented (8; 63; 122) but for which the mechanism(s) of action are still not well defined (45; 145).

How chemicals affect mosquitoes

Two of the most successful and widely used malaria control tools, IRS and LLINs, utilize residual insecticides to prevent disease transmission by controlling mosquitoes (40; 84; 126; 162). Remarkably, though, the various mechanisms by which these residual chemicals act upon vectors to prevent disease transmission are widely underappreciated (8; 63; 124). There has been a historical tendency to measure the value of an insecticide-based disease control intervention almost exclusively on the chemical's short-term toxic effect upon the target vector (63; 122): i.e., the only valuable outcome of an intervention is a dead vector. This conceptualization of how vector control can work is certainly valid, as decreasing vector longevity and overall vector abundance are two outcomes that can drastically, and quickly, reduce local vectorial capacity (161). Moreover, the toxic mechanisms by which insecticides kill their target organisms are also comparatively well understood. For example, the aforementioned pyrethroids, which are synthetic molecules derived structurally from naturally occurring botanical compounds called pyrethrins, are known to act as neurotoxins. In short, they bind voltage-gated sodium ion channels present in insect axons, prevent their closure and subsequent

repolarization, and result in paralysis (140; 141). Accordingly, pyrethroid resistance in mosquitoes is known to occur by at least two mechanisms: target site modifications (the so-called *kdr* mutations, which alter sodium ion channel configuration and prevent insecticide binding) (140) and metabolic detoxification (mostly by increased cytochrome P450 enzyme activity that metabolizes insecticide molecules into non-toxic subunits)(41).

Partly because insect death is such a clear, easily measureable endpoint produced by well understood mechanisms of action, the toxicity-centric approach to vector control has become a deeply entrenched public health paradigm despite the fact that numerous other effects of insecticides on mosquitoes, mainly behavioral modifications, have been recognized for decades (63; 122) and have been clearly shown to contribute to disease reduction in many settings (8; 122; 124).

Non-lethal effects of insecticides

Since their initial recognition, the non-lethal effects of insecticides on mosquito behaviors have been variously referred to by a confusing array of terms: behavioral avoidance, excito-repellency, contact irritability, deterrence, and inhibition, among others. However, what is probably the most complete contemporary understanding of these complex, interrelated behavioral responses comes from the probability model for the action of DDT, which was formally proposed by Roberts and others in 2000 and further developed by Grieco and others (2007) into a new classification system for IRS chemicals (Fig. 3) (63; 124). In basic terms, the scheme outlines three critical effects of chemicals on vectors: A) the spatial repellent (SR) effect, whereby an insect is stimulated to move away from the source of the chemical without making physical contact with it; B) the contact irritant (CI) effect, whereby an insect is stimulated to move away from the

source of the chemical after making physical contact with it; and C) the toxicant (TOX) effect, whereby the chemical produces knockdown or death in an insect (63; 124). An expanded concept of spatial repellency, which also includes chemical actions that interfere with host detection and/or otherwise disrupt the blood-feeding process, was established by WHO in 2013 to help determine guidelines for efficacy testing (172). Generally, this broader definition of SR put forth by WHO, which includes "...a range of insect behaviors induced by airborne chemicals that result in a reduction in human-vector contact and, therefore, personal protection (172)," is used herein, particularly when discussing experimental hut trials.

Importantly, not every public health chemical will elicit each kind of effect from every target species (13). Furthermore, their order of action (to the extent that more than one effect is present for a given vector-chemical interaction) depends on whether or not the concentration of active ingredient encountered by the target vector exceeds the threshold necessary for each specific response (63). It is telling that, even though these chemical thresholds are largely unknown, the contribution of spatial repellency to the prevention of disease transmission via the limiting of mosquito-human interactions has been demonstrated across many settings (8). This is perhaps best exemplified in the case of IRS with DDT and its repellant action against anopheline mosquitoes and resultant decreases in malaria incidence (122; 124-126).

The molecular basis of spatial repellency

Despite the importance of non-lethal behavioral modification of vectors for disease prevention, and the existence of a consumer market for insect repellent products worth several hundred million dollars a year (43), the molecular mechanisms that drive

spatial repellency in mosquitoes remain poorly understood. Current thought seems divided about whether or not the spatial repellency process is active, i.e. mosquitoes interact with volatilized repellent molecules and respond by orienting their movement down a concentration gradient away from the chemical source (113; 145), or if the process is passive, i.e. volatilized repellent molecules interfere with normal host detection pathways to mask the presence of a potential blood meal (44; 45). Regardless of which, or if, one of these models predominates, it would appear that olfactory pathways are often critical for the production of a spatial repellent response in mosquitoes (22), and that certain odorant receptor neurons are specifically configured to detect cognate repellent molecules (23; 79).

Less clear, however, is whether or not olfactory pathways are the only physiological drivers of spatial repellency behaviors in mosquitoes. Many insecticide compounds, including several pyrethroids, are known to induce irritant and/or hyperactive responses in mosquitoes at sub-lethal concentrations (34; 86; 132); this hyperactivity has been observed to promote the avoidance of impregnated nets (134). These particular behaviors are sometimes referred to as excito-repellency, and evidence suggests that these types of responses are caused by the same neurologically disruptive pathways that otherwise result in mosquito death when chemical exposures are more prolonged or of a higher dose (34; 81). When these behaviors are noted after a mosquito lands on a treated surface, it is said that the given chemical has contact irritant properties, as described above. However, whether or not these excito-repellency type responses might also be elicited by airborne, highly volatile active ingredients, and thereby

contribute to spatial repellency in some circumstances, is an interesting but so far unstudied question.

Clearly, a better understanding of the molecular and hereditary basis of spatial repellency behaviors in mosquitoes is needed. Such knowledge could help inform the rational development of new active ingredients (150) and, importantly, could also help predict how the prolonged use of spatial repellents might impact local vector populations over time: might there be selective pressures that result in the emergence of repellent insensitivity, similar or parallel to those selective pressures that so rapidly result in insecticide resistance?

RESEARCH GOALS AND BACKGROUND

The overarching goal of this dissertation research was to add to the body of knowledge examining the potential for push-pull strategies to control vectors of human disease. Key objectives included 1) evaluating the utility of the approach to control naturally occurring malaria vectors in a field environment in northern Belize, Central America and 2) exploring the heritability and molecular basis of spatial repellency behavior *in vitro* using dengue virus vectors colonized from this same region of northern Belize.

Belize

Belize is a small, tropical country located on the eastern coast of Central America bordered by the Caribbean Sea to the east, the Yucatán Peninsula of México to the north, and Guatemala to the west and south (Fig. 4). Ninety-five percent of Belizean territory (around 21,000 square kilometers) lies on the mainland of the Central American isthmus, while the other 5% (an additional 1,100 total square miles of land) is spread across

numerous islands and cayes in the Caribbean Sea (4). Formerly a British crown colony known as British Honduras, Belize gained independence from the United Kingdom in 1981 and is currently the only nation to be a full member of both the Central American Integration System (SICA) and the Caribbean Community (CARICOM), a position that reflects both its unique location and history.

Belize is divided into six administrative districts, often grouped into regions. Corozal and Orange Walk districts are in the northern region, the central region contains the districts of Belize and Cayo, and the southern region consists of Stann Creek and Toledo districts (Fig. 4). Ecologically, the country has a hot, tropical climate with distinct wet (approximately June to December) and dry (approximately February to May) seasons, though there are variations in weather patterns by region. The flat, swampy coastal plains of the north typically receive around 1,350 mm of rainfall annually, while similar coastal terrain in the south and central regions receive about three times as much rainfall, ~4,500 mm annually (1; 4; 58). The Maya Mountains, with a high elevation of 1,124 meters, roughly divide the north and south. Both large-scale and subsistence agriculture are key components of Belize's economy: sugar cane (*Saccharum* spp.) is the most common cash crop in the North, while banana and citrus plantations predominate in the south. Smaller-scale cultivation of maize, beans and rice are common throughout the country. Despite this agricultural activity and a long history of legal and illegal logging, much of mainland Belize remains covered by primary forest (31) and the country continues to maintain a high level of biodiversity (72).

The highly diverse population of Belize, which in 2013 was estimated to be around 334,000, reflects a mosaic of different cultures and ethnicities: Mestizo (50%),

Belizean Kriol (15%), Maya (11%), and Garifuna (6%) are the largest groups while significant populations of German Mennonites and South and East Asians are also relatively common (3; 4). Despite what is still a low population density (13.6/km^2, by far the lowest in Central America), net population growth has been relatively rapid over the last 10 years at almost 2% per year, and is aided both by regionally high fertility rates (26.4 births/1,000 population) and net immigration, mostly from neighboring Central American countries (3; 4). The population is roughly equally divided between urban (52%) and rural (48%) households (3). With a nominal gross *per capita* domestic product of $4,535 USD (76), Belize is classically described as a 'developing' nation and has a moderate 2012 Human Development Index (HDI) score of 0.702 (lower than the median HDI of 0.745 for Caribbean nations but higher than the median HDI of 0.680 for Central America) (151) and is one of 60 countries with currently active US Peace Corps Volunteers.

In Belize, there is a mix of private and public health facilities, but ultimately the Ministry of Health (MoH) is responsible for the oversight and delivery of health services to most of the population. Complex, urgent or advanced medical care is either unavailable or sought out abroad, mainly in Guatemala, México or the United States. Though exact numbers are not readily available, it is common for much of the population to receive periodic basic care from mobile clinics supported by numerous international NGOs and mission groups that are often active in the region. The National Vector Control Program (VCP) is the entity within the Ministry of Health that is responsible for the monitoring, control and prevention of vector-borne diseases. The VCP is headquartered in the capital city of Belmopan, but most activities are coordinated through

offices at each of the regional hospitals. There are numerous known and suspected vector-borne disease threats in Belize, but the limited resources available to the VCP are usually prioritized for the control of dengue virus vectors, nuisance biting mosquitoes and, to a lesser extent, malaria vectors.

Malaria in Belize

Currently, the WHO reports that 69% of the population of Belize live in areas of low transmission risk, while the remaining 31% live in malaria-free areas (Fig. 5A)(169). There were fewer than 100 cases of *P. vivax* malaria and a single case of *P. falciparum* (likely imported) officially reported to the MoH in 2012, all of which were reported to have been successfully treated with chloroqine (20). Around 92% of cases came from the southern districts of Stann Creek and Toledo, while the remaining 8% originated in the northern districts of Orange Walk and Corozal (20). This data is consistent with historical disease distribution patterns that have positively correlated malaria rates with total rainfall amounts, among other environmental and social factors (68). Malaria transmission and vector abundance are also highly seasonal in Belize, both increasing demonstrably during the rainy season, roughly June to December (60; 68; 119).

The current malaria situation in Belize is one of marked improvement over the past 20 years, and many within the MoH take pride in stressing that they are technically in the WHO 'consolidation phase' of malaria control and is headed towards the pre-elimination stage (personal observation). However, the history of malaria in Belize is a case study in erratic control efforts, close calls and disease re-emergence (5; 19; 20; 60; 68; 128). Before the routine spraying of houses with DDT began in the 1950's, malaria was perhaps the most significant health problem in Belize, afflicting nearly 50% of the

population and accounting for roughly 10 percent of all-cause mortality (128). By the early 1960's, widespread IRS campaigns had helped to reduce malaria rates dramatically (fewer than two dozen cases reported in 1961) and the country was considered well on its way to malaria elimination (25; 128). In the years that followed, vector control efforts varied in intensity and malaria incidence fluctuated. Although elimination was not achieved, overall transmission risk in Belize remained greatly reduced until the early 1990's (5; 19; 60; 128).

Precipitated by international pressures to ban the use of DDT, limited public health resources and a generally stressed vector control program, nationwide house spraying in Belize was abandoned in 1990 (5; 60; 68; 128). Not coincidently, malaria rates began increasing sharply in 1992 (5; 60; 128), and by 1994 incidence had increased two magnitudes of order to nearly 10,000 cases a year (68). This same year, the Pan American Health Organization classified the entire population of Belize to be at high risk for transmission (108): Belize was in the midst of a significant malaria outbreak.

Subsequent years have seen an emphasis on case identification (both passive and limited active case detection) and aggressive treatment, bi-annual house-spraying with deltamethrin in the ten most at-risk communities of each district, and ULV spraying at every incident locality (2). These actions that have helped to reverse the outbreak trends and lead to the more encouraging situation of today. However, waning resources, shifting priorities and a regionally disjointed commitment to malaria control, combined with the natural abundance of multiple competent vectors and the commonplace movement of people to and from neighboring malaria endemic countries, create a level of concern that Belize is continually at risk for another outbreak.

The ecology of malaria transmission in Belize is varied, the result of the ecologically diverse landscape of the country. Fourteen species of anopheline mosquitoes have been reported from Belize (173), but only four of these are likely vectors of human malaria (64): *An. vestitipennis* and *An. albimanus* throughout the coastal wetlands and swamps; *An. darlingi* near its riverine habitats, especially in the central and southern regions; and *An. pseudopunctipennis* in rock pools and streams of the central higher elevations. The first two vectors on this list, *An. vestitipennis* and *An. albimanus*, are particularly abundant at and around the field site outside of Progresso Village in Corozal district that was selected for our experimental huts (described in greater detail in later chapters) and were the target vectors of the experimental push-pull intervention evaluated here.

An. vestitipennis

In Belize, *An. vestitipennis* breeds predominately in shaded swamps and flooded forests, often in association with cattail (*Typha* spp.) and other tall dense macrophytes (118). Not surprisingly, population densities of *An. vestitipennis* are highest during the rainy season when larval habitat is most abundant (60). This species has been incriminated as a principal vector of malaria in Belize based on minimum field infection rates (12) and its endophagic, anthropophagic behavioral tendencies (19; 60). In previous experimental hut studies in Belize, *An. vestitipennis* were shown to enter huts primarily through open windows, to bite frequently throughout the night, and to be susceptible to the spatial repellent, contact irritant and toxicant stimuli of DDT (10; 19; 61).

An. albimanus

An. albimanus also breeds in the freshwater swamplands and flooded savannahs of northern Belize, but prefers areas of greater sunlight and is often found in association with cyanobacterial mats (119; 120). *An. albimanus* populations are also seasonal, and are naturally highest during the rainy season but can also be strongly associated with the cultivation of rice during the dry season in Belize (60). *An. albimanus* has long been considered an important vector in Central America and the Caribbean (137), and has been incriminated as transmitting malaria in Belize and southern México (12; 115). However, because of its relatively exophagic and zoophagic behavior patterns, high population densities may be required for this species to efficiently transmit malaria within a community (19; 60; 64). In previous experimental hut studies in Belize, *An. albimanus* was also shown to enter huts primarily through open windows, but showed a distinct exophagic preference with a bimodal biting pattern and peaks of activity early in the evening and again shortly before sunrise (10; 19; 61). This species has also demonstrated behavioral and toxic susceptibilities to DDT (19).

Dengue Fever in Belize

Dengue virus infection is now endemic throughout the entire country of Belize (Fig. 5B): more than 1,500 confirmed cases *per annum* were reported for the years 2009-2012 with sporadic (less than 10) reports of hemorrhagic disease (20). Accordingly, dengue case monitoring and vector control are considered high priorities in both the tourism and public health sectors (152). Though *Ae. albopictus* (Skuse) has been established in Belize, it's role in dengue virus transmission is unknown (155) and

virtually all transmission in Belize is thought to occur via the highly anthropophilic and anthropophagic *Ae. aegypti.*

In Belize, the national Vector Control Program also coordinates dengue control activities, which include public outreach, passive case identification, source reduction, larvaciding with temephos, seasonal truck-mounted ULV spraying with malathion and hand-held thermal fogging with malathion or lamda-cyhalothrin at every incident household.

Ae. aegypti

Globally considered the most prevalent vector of dengue virus, *Ae. aegypti* is highly adapted to an anthropophilic lifestyle, preferring to feed on humans and during the day and easily breeding in a variety of man-made habitats including discarded tires, flower pots, drainage and water collection systems (27) . Although rains and flooding can temporally increase *Ae. aegypti* densities, the vector is less susceptible to the seasonal fluctuations of wet and dry season habitat availability (165) and can be found year-round in Belize (personal observation). Because of its global public health importance as the principal vector of dengue virus, yellow fever virus and chikungunya virus, and the fact that its natural life history allows it to be easily maintained in colony, *Ae. aegypti* has also become one of the most widely used model organisms in medical entomology and vector biology research (33).

Transfluthrin

Transfluthrin (2,3,4,6-tetrafluorobenzyl (1R)-trans-3-(2,2-dichlorovinyl)-2,2-dimethyl cyclopropanecarboxylate), a volatile synthetic pyrethroid with both contact toxicant and SR efficacy against several classes of arthropod pests including mosquitoes

(48; 105; 107; 110), was selected as the test repellent for these studies. In addition to having proven SR efficacy, transfluthrin is easily deployed in experimental settings using manufacturer's (S.C. Johnson and Son, Inc., Racine WI) recommendations and industry guidelines. Briefly, reagent grade (99%, unformulated) transfluthrin is diluted in acetone and used to treat strips of cloth from which the active ingredient then emanates passively into the surrounding airspace. Treatment and control cloth strips (treated with acetone only) can then be secured inside an experimental hut or used in an *in vitro* behavioral bioassay.

Experimental Huts

Experimental huts have proven to be an important tool for evaluating the behavioral ecology of naturally occurring malaria vectors in their native habitats (136), including assessments of the impact of insecticides and other chemicals on the resting, entry, feeding and exit patterns of mosquitoes (61; 122). In order to improve the relevancy of results obtained with experimental huts to real world situations, it is important that hut designs and construction materials closely resemble the housing conditions typical in the communities around where the studies are occurring (9; 60; 136). In Belize, specially constructed experimental huts made of locally acquired materials and patterned after a typical rural Belizean homes (Fig. 6) have been used successfully to evaluate the behaviors of *An. vestitipennis* (10; 19; 61), *An. albimanus* (10; 19) and *An. darlingi* (9; 11; 130).

Mosquito Traps

The trapping of mosquitoes in general, and *Anopheles* spp. in particular, is often described as difficult (49; 174). Though there is broad interest in developing tools and

strategies able to trap large numbers of mosquitoes (83), to date the success of trapping schemes to reduce local densities of host-seeking mosquitoes has been mostly elusive, even when carried out on mass scales using multiple, sophisticated traps (35; 71; 138). Regardless, there is a clear role for traps in sampling local mosquito diversity and estimating relative abundance, and several trap types are routinely integrated into research and public health surveillance activities. Among these established traps, the CDC Miniature Light Trap Model 512 (CDC LT) (John W. Hock Company, Gainesville, FL) and the BG Sentinel™ trap (BGS) (Biogents, A.G., Regensburg, Germany) are among the most frequently used. Both traps are widely available, field deployable, and easily baited. The CDC LTs remain an industry standard trap used to sample and capture *Anopheles* spp. mosquitoes for public health and vector research applications (135; 170). The BGS trap, though specifically designed to capture *Aedes* spp. mosquitoes, has shown some efficacy at trapping *Anopheles* spp. and has been previously integrated into field experiments demonstrating proof of concept of a push-pull strategy for the control of dengue virus vectors (7; 74; 132; 133).

The HITSS Bioassay

The high throughput screening system (HITSS) is a bioassay developed by Grieco and others to screen chemicals for effects on arthropod behavior (Fig. 7) (65). The interchangeable, modular setup allows for the evaluation of three distinct outcomes of chemical exposure: toxicity (TOX), contact irritancy (CI) and spatial repellency (SR). Though not previously reported, the modular nature of the HITSS assay device also allows for the ability to collect experimental mosquitoes for further testing and breeding after their behavioral responses to various chemicals have been observed and recorded.

This would allow for investigations into the behavioral plasticity of SR behaviors via re-testing experimental mosquitoes at subsequent time points (143), and into the inheritance of SR behaviors via selective breeding of responders and non-responders (144). Though this type of *in vitro* testing is only amenable to species that are easily colonized and reared to large numbers, this kind of novel application of the HITSS bioassay system could yield valuable insights into the hereditary and molecular nature of complex spatial repellency behaviors in certain mosquito vectors of human disease.

SPECIFIC AIMS AND HYPOTHESES

In order to address the key research objectives outlined above, two sets of specific aims were established.

Specific Aim 1: In the Field

Evaluate the efficacy of a prototype push-pull intervention to control two naturally occurring vectors of human malaria, *Anopheles vestitipennis* (Dyar and Knab) and *An. albimanus* (Wiedemann), using experimental huts in northern Belize, Central America.

Hypothesis: A combined push-pull intervention would result in greater reductions in mosquito entry into experimental hut windows and increased capture of mosquitoes in outdoor traps compared to the use of either the push or pull intervention alone.

Sub aim 1a: Characterize baseline mosquito densities and hut entry patterns at the experimental field site.

Sub aim 1b: Explore outdoor mosquito trap dynamics at the field site.

Sub aim 1c: Observe the effect of transfluthrin treatment on *Anopheles* hut entry behaviors.

Sub aim 1d: Evaluate the efficacy of a combined push-pull approach to reduce the number of indoor *Anopheles* and increase the capture of *Anopheles* in outdoor traps.

Specific Aim 2: In the Lab

Use an *in vitro* behavioral bioassay to investigate the plasticity, heritability and molecular basis of spatial repellency behaviors in a Belizean strain of *Ae. aegypti*.

Hypothesis: The *in vitro* spatial repellency response to transfluthrin observed in *Ae. aegypti* is a heritable trait associated with the presence of specific odorant receptor genotypes and/or insecticide susceptibilities.

Sub aim 2a: Characterize the plasticity of *in vitro* SR behavior.

Sub aim 2b: Determine the heritability of *in vitro* SR behavior.

Sub aim 2c: Explore differences among SR responders and SR non-responders from selectively bred mosquito strains, with a focus on odorant receptor gene profiles and/or insecticide susceptibility patterns.

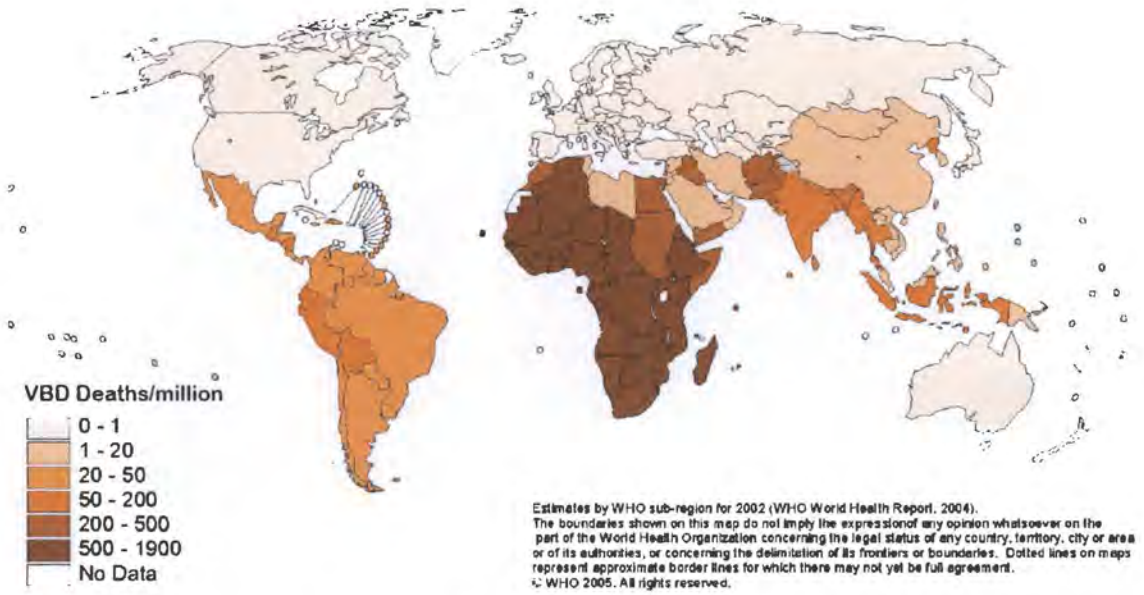


Figure 1. The global distribution of deaths caused by vector-borne diseases. Adapted from the World Health Report, WHO, Geneva, 2004.

Table 1. List of insecticides currently recommended by the World Health Organization (WHO) for public health applications

Insecticide	Class	Recommended Use[1]	WHO hazard classification[2]
Bendiocarb	Carbamate	IRS	II
Propoxur	Carbamate	IRS	II
DDT	Organochloride	IRS	II
Fenitrothion	Organophosphate	IRS, Space Spray	II
Malathion	Organophosphate	IRS, Space Spray	III
Pirimiphos-methyl	Organophosphate	IRS, Space Spray	III
α-Cypermethrin	Pyrethroid	IRS, LLIN	II
Bifenthrin	Pyrethroid	IRS	II
Cyfluthrin	Pyrethroid	IRS, LLIN, Space Spray	II
Deltamethrin	Pyrethroid	IRS, LLIN, Space Spray	II
Etofenprox	Pyrethroid	IRS, LLIN, Space Spray	U
λ-Cyhalothrin	Pyrethroid	IRS, LLIN, Space Spray	II
Permethrin	Pyrethroid	LLIN, Space Spray	II
D-Phenothrin	Pyrethroid	Space Spray	U
Resmethrin	Pyrethroid	Space Spray	III

[1]Uses include: IRS = Indoor Residual Spray; LLIN = Long Lasting Insecticide-Treated Bed Nets; Space Spray = Aerosol Spraying or Thermal Fogging

[2]Class II, moderately hazardous; class III, slightly hazardous; class U, unlikely to pose an acute hazard in normal use

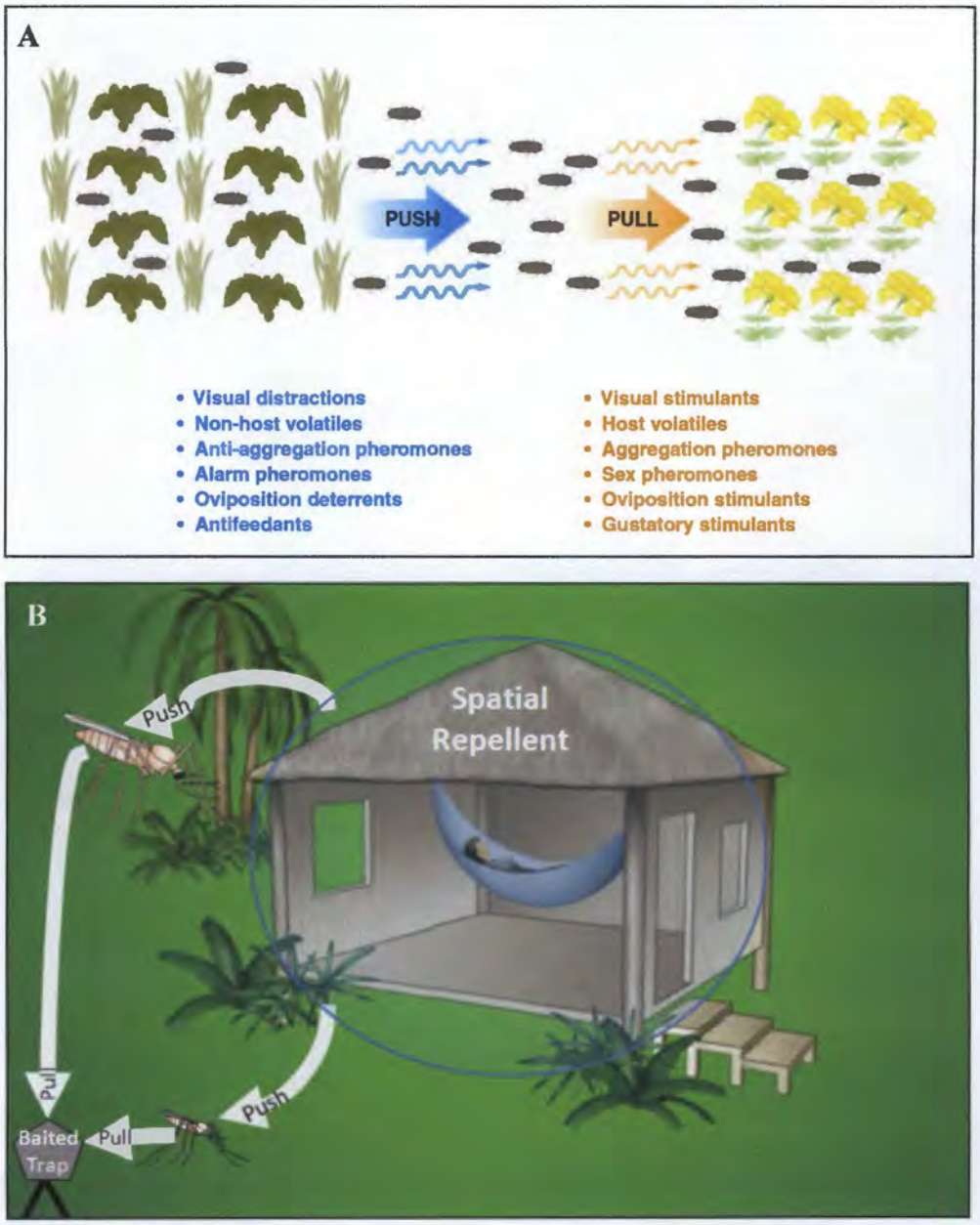


Figure 2. Push-pull strategies of arthropod control in agriculture (in use) and public health (theoretical).
(A) An established model for the protection of crops is illustrated. From Cook et al., 2007. (B) A theoretical application of a push-pull strategy for the control of malaria vectors. Adapted from Roberts et al., 2010.

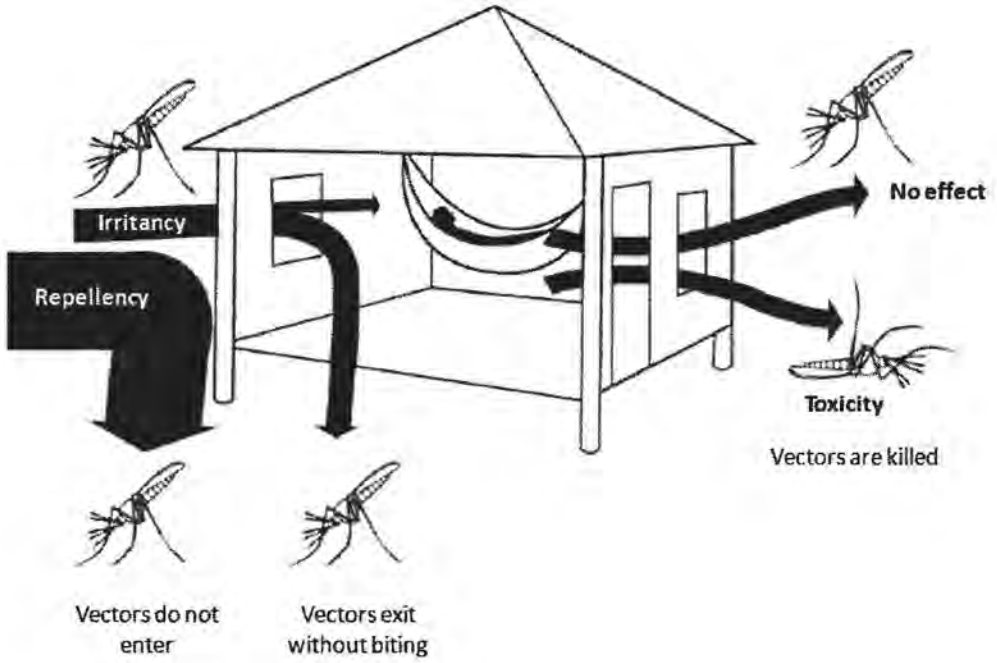


Figure 3. Public health insecticides exert multiple actions on disease vectors. The example of a house treated by indoor residual spray with DDT for malaria prevention is shown. In this case, the repellency, irritant and toxicant actions each serve to reduce disease transmission. Adapted from Roberts et al., 2010.

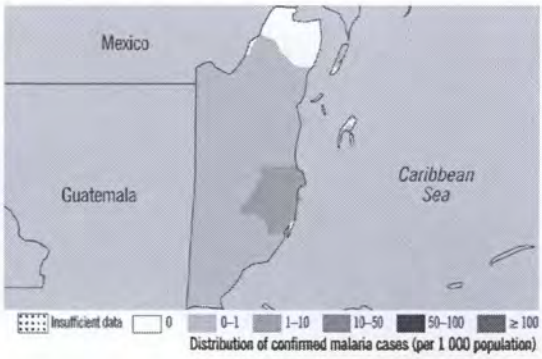
Figure 4. The location of Belize in Central America and the geopolitical divisions of Belize.
The study areas for this research were located in the northern districts of Corozal (experimental hut studies) and Orange Walk (dengue vector collections). Adapted from http://en.wikipedia.org.

A

Belize

Phase: Control. Coverage: In 2010, IRS was sufficient to protect 23% of the population at high risk.

I. EPIDEMIOLOGICAL PROFILE		
Population (UN Population Division)	2010	%
High transmission (≥ 1 case per 1000 population)	0	0
Low transmission (0-1 cases per 1000 population)	215 000	69
Malaria-free (0 cases)	96 600	31
Total	311 600	



B

Figure 5. Belize is endemic for both (A) malaria and (B) dengue fever. (A) From the World Malaria Report 2012, WHO. (B) From Health Information for International Travel (The Yellow Book) 2006, US CDC.

Figure 6. A Belizean experimental hut used for studying the behavioral ecology of malaria vectors.

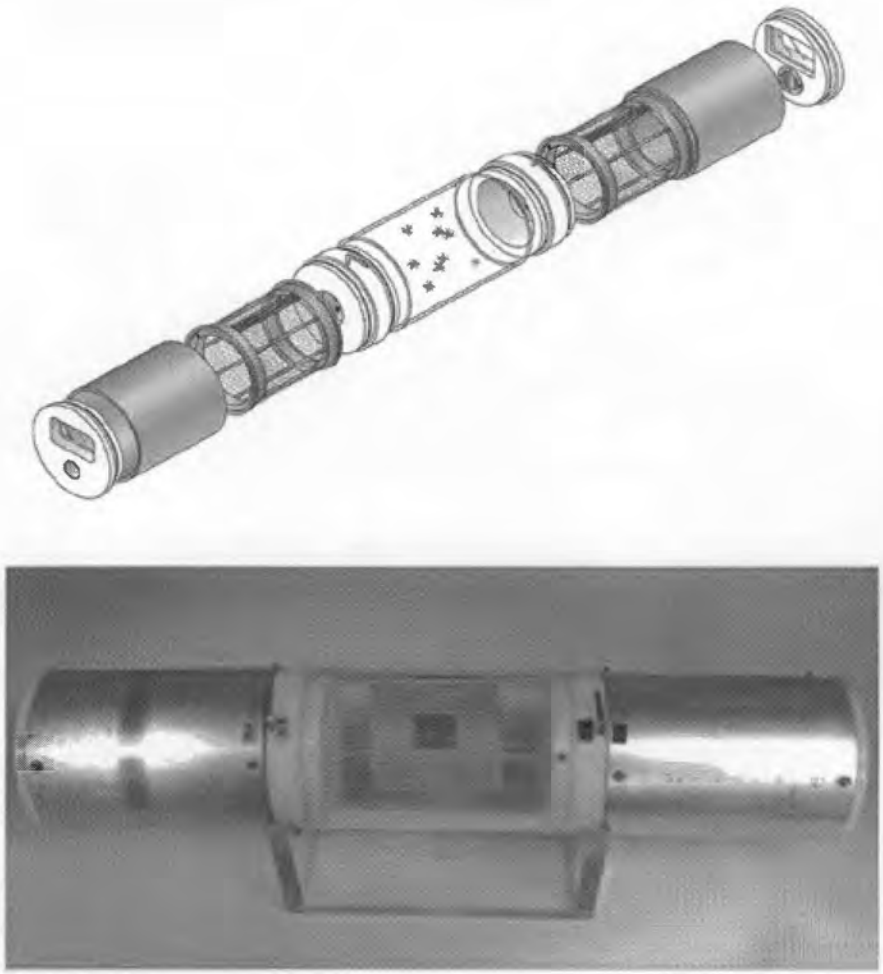


Figure 7. The high throughput screening system.
Shown as used to investigate *in vitro* spatial repellency behaviors in disease vectors. Diagram adapted from Grieco et al., 2005.

CHAPTER 2: A comparison of Two Commercial Mosquito Traps for the Capture of Malaria Vectors in Northern Belize, Central America[2]

ABSTRACT

To achieve maximum success from any vector control intervention, it is critical to identify the most efficacious tools available. The principal aim of this study was to evaluate the efficacy of two commercially available adult mosquito traps for capturing *Anopheles albimanus* and *An. vestitipennis*, two important malaria vectors in northern Belize, Central America. Additionally, the impact of outdoor baited traps on mosquito entry into experimental huts was assessed. When operated outside of human-occupied experimental huts, the CDC miniature light trap, baited with human foot odors, captured significantly greater numbers of female *An. albimanus* per night (5.1 ± 1.9) than the Biogents Sentinel™ trap baited with BG-Lure™ (1.0 ± 0.2). The two trap types captured equivalent numbers of female *An. vestitipennis* per night, 134.3 ± 45.6 in the CDC trap and 129.6 ± 25.4 in the Sentinel trap. When compared to a matched control hut using no intervention, the use of baited CDC light traps outside an experimental hut did not impact the entry of *An. vestitipennis* into window interception traps, 17.1 ± 1.3 females per hour in experimental huts vs. 17.2 ± 1.4 females per hour in control huts. However, the use of outdoor baited CDC traps did significantly decrease the entry of *An. albimanus* into window interception traps from 3.5 ± 0.5 females per hour to 1.9 ± 0.2 females per hour.

[2] The majority of Chapter 2 has been accepted for publication as:
Wagman J, Grieco J, Bautista K, Polanco J, Briceño I, King R and Achee N. 2014. A Comparison of Two Commercially Available Adult Mosquito Traps for the Capture of Malaria Vectors in Northern Belize, Central America. *J Am Mosq Control Assoc* 30(3):000 (In Press).
JW assumes sole responsibility for the work presented here. JW designed the study, performed the experiments, interpreted the data and wrote the manuscript. JG and NA contributed to the design of the study, interpretation of the data, and revision of the manuscript. KB, JP, IB, and RK contributed to acquisition of the data and revision of the manuscript.

These results support existing knowledge that the underlying ecological and behavioral tendencies of different *Anopheles* species can influence trap efficacy. Furthermore, these findings will be used to guide trap selection for future push-pull experiments to be conducted at the study site.

Key Words
Belize, push-pull strategy, *Anopheles albimanus, Anopheles vestitipennis*, outdoor traps

INTRODUCTION

Operational realities such as the management of insecticide resistance and the need to target the behavioral patterns (e.g. outdoor, early evening and/or day-biting) of a wide range of vector species are limiting the effectiveness of traditional vector control tools such as indoor residual spraying (IRS) and long-lasting insecticide nets (LLINs) in many malaria endemic settings (13; 14; 47; 63; 97). It is not surprising, therefore, that the development of novel vector control strategies has been identified as a top priority within the global health community (47; 85; 149; 159; 167). One novel strategy under consideration is a push-pull method, whereby the complementary actions of spatial repellents (which 'push' or deter vectors from entering treated spaces) and mosquito traps (which 'pull' or remove vectors from a given outdoor area) are used simultaneously to decrease the probability of human exposure to mosquito bites. Such an outcome could serve to prevent pathogen transmission in a variety of settings (8; 38; 82; 111).

As with all vector control interventions under development, identifying the most efficacious tools, and the challenges to their implementation, is critical to achieving maximum success. This includes a thorough understanding of local disease transmission dynamics, such as recognition of the primary vector species, target vector behavior patterns and, ideally, field evaluation to drive optimization. The current study represents

one component of a larger field project focused on the evaluation of a push-pull strategy for the control of malaria vectors in northern Belize, Central America, where *Anopheles vestitipennis* and *An. albimanus* are both known to be regionally important vectors (12; 55; 64; 137; 175). Importantly, each species has also been characterized, in Belize, to exhibit different behavioral profiles: *An. vestitipennis* is known to be more highly endophagic and anthropophagic, while *An. albimanus* tends to be more highly exophagic and zoophagic (19; 62; 64).

Trapping malaria vectors has often been described as difficult (49; 174). However, several traps have been successfully integrated into research and routine surveillance activities. Among these established traps, the CDC Miniature Light Trap Model 512 (CDC LT) (John W. Hock Company, Gainesville, FL) and the BG Sentinel™ trap (BGS) (Biogents, A.G., Regensburg, Germany) were selected for evaluation in the current study. Both traps are widely available, field deployable, and easily baited. The CDC LTs remain an industry standard trap used to sample and capture *Anopheles* spp. mosquitoes for public health and vector research applications (135; 170). The BGS trap, though specifically designed to capture *Aedes* spp. mosquitoes, has shown some efficacy at trapping *Anopheles* spp. and has been previously integrated into field experiments demonstrating proof of concept of a push-pull strategy for the control of dengue virus vectors (8; 74; 132; 133).

Specific objectives of the experiments described here included quantifying 1) the efficacy of the CDC light trap and BG-Sentinel trap at capturing the target vectors *Anopheles vestitipennis* and *An. albimanus* and 2) the effect of baited traps in the peridomestic area on mosquito entry into human-occupied experimental huts.

MATERIALS AND METHODS

Field Site

The study site was located in an open pasture near Progresso village in the Corozal District of Northern Belize (18°11'52"N, 88°26'18"W), surrounded by freshwater swamplands and several permanent lagoons (Fig. 8). The rainy season in northern Belize typically lasts from May to December, when the region experiences average rainfall of around 200 mm/month and *Anopheles* spp. mosquito densities are highest (58; 66).

Experimental Huts

Two identical experimental huts, located 50 meters apart along a straight north-south transect, were constructed on site (Fig. 9). Based on a previously described portable hut design (9), huts were made in a style typical of regional homes using locally acquired materials. Briefly, each structure measured 3.6 square meters and was constructed of an untreated pine lumber frame with plywood walls and flooring and a corrugated tin roof. Both huts were fashioned with one 182 cm by 76.2 cm door cut into the eastern facing wall, and each of the three remaining walls contained one 76.2 cm^2 window built to accommodate interception traps.

Mosquito Lures, Trap Placement and Outdoor Collections

Two types of mosquito lures were used in conjunction with the outdoor traps. First was the BG-Lure™, a blend of components including lactic acid, ammonia, caproic acid and other fatty acids (154). Although specifically intended to capture *Aedes* spp. in conjunction with the BGS trap, there is some evidence that the BG-Lure can attract *Anopheles* spp. as well (93), and it is recommended by the manufacturer to increase BGS

trap yields of other mosquitoes, including *Anopheles* spp. (21). The BG-Lure was handled and used according to manufacturer's instructions for use in tropical climates. The second lure consisted of human foot residues emanating from worn cotton socks placed on top of the CDC LT rain guard (101; 133). Prior to use in mosquito collections, sock lures were worn one pair at a time for 12 hours by the same individual during periods of roughly equal activity (i.e. daily preparations at the field site or during the overnight collections). For use as mosquito lure, a randomly selected pair of worn socks aged between 24 and 72 hours was placed at each CDC LT. Each pair of socks was used for two collections before replacement with a more recently worn pair. When not in use, sock lures were kept at ambient temperature in sealed plastic bags away from direct sunlight. Baited traps were positioned outside each window of an occupied experimental hut and operated according to manufacturer's instructions: CDC traps were hung 2m above ground while BGS traps were positioned parallel to the hut platform (1m above ground). Both trap types were set at a 1m distance from the exterior hut wall (Fig. 9B and 9C). Outdoor traps were positioned, baited and switched on 30min prior to sunset (~1730h) and operated continuously to just after sunrise (~0600h). Collection bags were removed and replaced every 60 min during each 12h collection period.

Indoor Collections

Window interception traps were based on the designs of Muirhead-Thomson (95) and Grieco and others (61) and fitted onto each experimental hut window (Fig. 9A). Collections from interception traps were conducted every 30min during a 12h sampling period (1800h-0600h). Collections were further divided into four 3h shifts to allow for equal rotation of collectors. The door of each hut remained closed during the entire

sampling period so that the windows, with attached interception traps, represented the only portals of hut entry for host-seeking mosquitoes. To facilitate mosquito collection, trap portals were temporarily closed with 3in. polyurethane foam (Landy's and Sons, Ltd., Orange Walk Town, Belize) at the top of each collection interval and immediately re-opened afterwards. Thirty minutes before dusk (~1730h), a two-person collection team entered each hut to provide indoor host cues and prepare for mosquito collections. Starting at 1800h, one collector in each hut aspirated all mosquitoes from the interception traps for a total of 15 minutes (i.e., one 5 min interval per window), while the second collector rested. Collectors rotated capture and resting activities at the conclusion of every 3h shift. Collected mosquitoes were placed into plastic cups individually labeled by hut, time and unique window code and killed using acetone vapors.

Study Design

To control for bias in mosquito capture efficiency, collector attractiveness between teams and/or mosquito abundance by hut locations, a Latin square study design was employed such that each trap type, lure and collection team was rotated between each hut. Initial window interception trap collections conducted without any experimental interventions from 28 September to 06 October, 2011, indicated high mosquito densities and excellent baseline comparability between huts, collection teams, and nights with no significant differences (ANOVA, $\alpha = 0.05$) observed in terms of mosquitoes collected (Fig. 10). Three separate experiments were then conducted. The first, from 17 to 27 October, 2011, was a head-to-head comparison of the efficacy of CDC LTs using the literature-recommended sock lure and BGS traps using the manufacturers specified BG-Lure, operated simultaneously at different occupied huts over four nights (a total of 12

trap-nights for each outdoor trap type). The second experiment, also over four nights, was conducted from 03 to 19 November, 2011, and measured the impact of baited CDC LTs on mosquito hut entry compared to an untreated control (a total of 12 interception trap-nights each for light trap intervention and control). The final experiment was conducted from 10 to 28 January 2012 to compare the efficacy of CDC LTs baited with either sock lure or BG-Lure (6 trap-nights for each lure type). All mosquitoes captured in the study were identified to subfamily, with anopheline mosquitoes further identified to species using a site-appropriate morphological key (173).

Data Analysis

Unless otherwise stated, the geometric means of mosquitoes captured are presented with the standard error of the means. Raw data in the form of total numbers of mosquitoes captured were $\log_e$ transformed and means, standard errors of the means and confidence interval endpoints were calculated and then back-transformed to obtain the geometric means. Means were compared via Student's t-test ($\alpha = 0.05$) using Excel 2007 (Microsoft, Redmond WA) and SPSS Statistics 20 (IBM, Armonk NY). For the first experiment, the difference in the mean number of mosquitoes collected from CDC LTs and BGS traps was calculated. In the second experiment, the impact of the presence of outdoor CDC LTs on mosquito entry into experimental huts was evaluated by comparing the mean number of mosquitoes collected from interception traps in control huts (no CDC LTs) and from interception traps in huts with baited CDC LTs hanging outside the windows. Lastly, a head-to-head comparison of lure types used in conjunction with CDC LTs calculated the difference in mean number of mosquitoes collected form traps using human foot odors and traps using the BG-Lure. Weather data to include indoor and

outdoor temperature, humidity, wind speed and direction, were recorded using HOBO® Pro Series Weatherproof Data Loggers (Forestry Suppliers Inc., Jackson, MS) and a DiC-3 handheld anemometer (Maximum Weather Instruments, New Bedford, MA).

RESULTS

A total of 28,946 mosquitoes were collected during 16 all night (12h) collections. In all, five *Anopheles* species were identified: 20,560 *An. vestitipennis* (71.0%); 1,805 *An. albimanus* (6.2%); 1,319 *An. crucians* (4.6%); 1,021 *An. punctimacula* (3.5%); and 796 *An. gabaldoni* (2.7%) (Fig. 11A). Culicine mosquitoes, which were not able to be identified to species level, accounted for the remaining 11.9% of the total caught and included predominately *Culex* spp., *Psorophora* spp., and *Mansonia* spp. (Fig. 11A). The proportional abundance of each species captured in the three trap types differed: *An vestitipennis* made up 73.6% (19,025/25,833), 72.4% (627/865) and 43.6% (774/1,773) of all mosquitoes collected from window interception traps, BGS traps and CDC LTs, respectively (Fig. 11B). Conversely, *An. albimanus* accounted for 6.7% (1,754/25,833) and 1.5% (28/1,773) of all mosquitoes from window interception traps and CDC LTs, respectively, and less than 0.5% (4/865) of all mosquitoes captured in BGS traps (Fig. 11B).

Results from trials comparing the efficacy of CDC LTs and BGS traps indicate that there were no significant differences in the nightly average numbers of mosquitoes entering the experimental huts based on outdoor trap type (Table 2). Outdoors, the nightly mean *An. vestitipennis* captured in CDC LTs baited with human foot odors (134.3 ± 45.6) did not significantly differ from the mean captured in BGS traps baited with the BG-Lure (129.6 ± 25.4) over the four night trial (Fig. 12). However, the CDC LTs did

capture a greater number of *An. vestitipennis* than the BGS traps on three of the four nights. For *An. albimanus*, the CDC LTs captured an average of 5.1 ± 1.9 mosquitoes per night, significantly more ($P<0.05$) than the BGS traps that captured 1.0 ± 0.2 mosquito per night (Fig. 12). CDC LTs also captured greater nightly averages of *An. crucians* (43.9 ± 40.7 vs. 3.8 ± 0.9) and *An. punctimacula* (77.3 ± 79.8 vs. 6.9 ± 1.0) than did BGS traps (Table 2). When CDC LTs were used in conjunction with two different lure types, data showed human foot residues on socks to attract significantly more *An. vestitipennis* (2.6 ± 2.4 per night) than when the BG-Lure was used (0.0 per night) (Table 3). There were no *An. albimanus* collected during this particular experiment.

Results from the evaluation of sock-baited CDC LTs on hourly mosquito hut entry suggest that the presence of baited CDC LTs outside of windows did not significantly reduce the numbers of *An. vestitipennis* collected per hour in window interception traps compared to control huts with no outdoor trap treatment (17.1 ± 1.3 CDC LTs vs. 17.2 ± 1.4 Control) (Table 4, Fig. 13). Additionally, there was no impact on the time of peak entry, or general entry pattern, observed for *An. vestitipennis* (Fig. 14). On the other hand, significantly fewer *An. albimanus* were captured per hour from portals of entry when CDC LTs were positioned outdoors compared to a control (1.9 ± 0.2 vs. 3.5 ± 0.5) (Table 4, Fig. 13). Further analysis based on time of collection showed that the reduction in *An. albimanus* entry was statistically significant ($\alpha < 0.05$) only during the early evening, within three hours of sunset (Fig. 14).There was also a significant reduction in *An. punctimacula* entry in the presence of a baited CDC LT (0.3 ± 0.3 vs. 0.2 ± 0.0) (Table 4).

Meteorological data (Table 5) showed that winds were predominantly out of the northeast and generally calm with nightly maximum speeds consistently occurring during the first 3 hours of the collection period, averaging 2.4 km/h (± 0.75 km/h). Outdoors, the average temperature was 21.6°C (± 3.1 °C) with a range from 28.3°C to 17.5°C while the average relative humidity was 94.7%, (± 2.6%). Indoors, temperature and relative humidity measurements were not significantly different (P=0.05) between huts and data points were therefore pooled from both structures: indoor temperatures averaged 23.4°C (± 2.9 °C), ranging from 29.9°C to 19.1°C, and the average indoor relative humidity measured 81.6% (± 6.4%). Though rain showers were common during daytime hours, no rainfall occurred during these overnight collection periods.

DISCUSSION

The first objective of the current study was to quantify the efficacy of two outdoor traps, used in conjunction with different lures, in capturing two important malaria vectors in northern Belize, *An. vestitipennis* and *An. albimanus*. The goal was to determine which trap would be most appropriate for future use in combination with an indoor spatial repellent for the evaluation of a push-pull mosquito control strategy. Our findings show both the CDC LT and BGS trap captured target *Anopheles* species, but differences in density and proportion of each species existed by trap type. These results reflect similar findings from other studies that have also shown species composition, in terms of proportions of total catches, is dependent on the specific trap and lure used (49; 92; 104; 174). Although both CDC LTs and BGS traps collected equivalent numbers of *An. vestitipennis*, the CDC LT captured significantly more *An. albimanus* females. Additionally, during experiments performed at the end of the 2011 rainy season with

lower overall mosquito densities, the use of the BG-Lure with CDC LTs failed to capture any *Anopheles* spp. mosquitoes and was clearly outperformed by CDC LTs using the human foot odor bait. This suggests that the CDC LT baited with dirty socks is suitable for subsequent push-pull experiments at this study site. However, a more comprehensive investigation of lure and trap type might be warranted for providing valuable insight into lure efficacy across different mosquito species.

A second objective was to quantify the effect of a baited CDC LT hanging in the peridomestic area on mosquito entry into an occupied experimental hut. Baited light traps are often used for sampling local mosquito populations (83; 135; 170) but are not generally effective as standalone interventions to control them (83; 121). Accordingly, it was somewhat unexpected that the use of CDC LTs outside experimental hut windows would have an impact on mosquito entry into the hut, as measured by collections from window interception traps. However, there was a significant reduction in early evening *An. albimanus* entry. Caution should be used not to over interpret this reduction (an average of 8 fewer mosquitoes during the first three hours after sunset) as epidemiologically significant. It is, however, important to note that the decreased entry was most prominent during the time of night that corresponds to peak feeding behaviors previously observed for *An. albimanus* in Belize (10; 11; 19; 127). This suggests that an outdoor trap alone could negatively impact indoor densities of this important malaria vector, thereby disrupting human vector contact. Interestingly, there was no corresponding effect of outdoor CDC LTs on *An. vestitipennis* entry, as the numbers of *An. vestitipennis* females collected from window interception traps was the same at control and experimental huts. These results may indicate that an outdoor baited trap

more effectively targets the exophagic *An. albimanus*, whose outdoor host-seeking behaviors are more likely to be impacted by the outdoor lure, while the more endophagic *An. vestitipennis*, with a stronger affinity for feeding indoors, appears more likely to bypass an outdoor trap and proceed to enter an occupied structure to feed.

In conclusion, findings from the experiments described herein support existing knowledge that the underlying ecological and behavioral tendencies of different *Anopheles* species can profoundly influence trap efficacies. Such information underlies the importance of characterizing disease transmission dynamics at the local level in order to drive the development and optimization of vector control strategies. At this site in northern Belize, CDC LTs baited with human foot odors and BGS traps baited with BG-Lure were equally effective in collecting the malaria vector *An. vestitipennis* when deployed outside of occupied experimental huts, but the CDC LT was clearly more efficacious in collecting the sympatric vector *An. albimanus*. Accordingly, the CDC LT baited with foot odors is the better trap choice for further studies of malaria vector control interventions at the site.

ACKNOWLEDGMENTS

We would like to thank Dr. Michael Pitts and Dr. Aisha Andrewin of the Belize Ministry of Health for their review and support of the study. We also thank Susana Castillo and Ian Briceño of the Belize Vector and Ecology Center for their invaluable assistance in hut construction and collection and processing of mosquito samples. Special acknowledgement to Mr. Albert Burns for the use of his land for the study site. Dr. Philip Coyne, Dr. Stephen Davies, Dr. D. Scott Merrell, and Dr. Cara Olsen of the Uniformed

Serviced University of the Health Sciences also provided valuable input and guidance into the experimental design and analysis.

The opinions contained herein are the private views of the authors and are not to be construed as official or reflecting the views of the Department of Defense or the Uniformed Services University of the Health Sciences. The use of commercial names does not constitute product endorsement or recommendation.

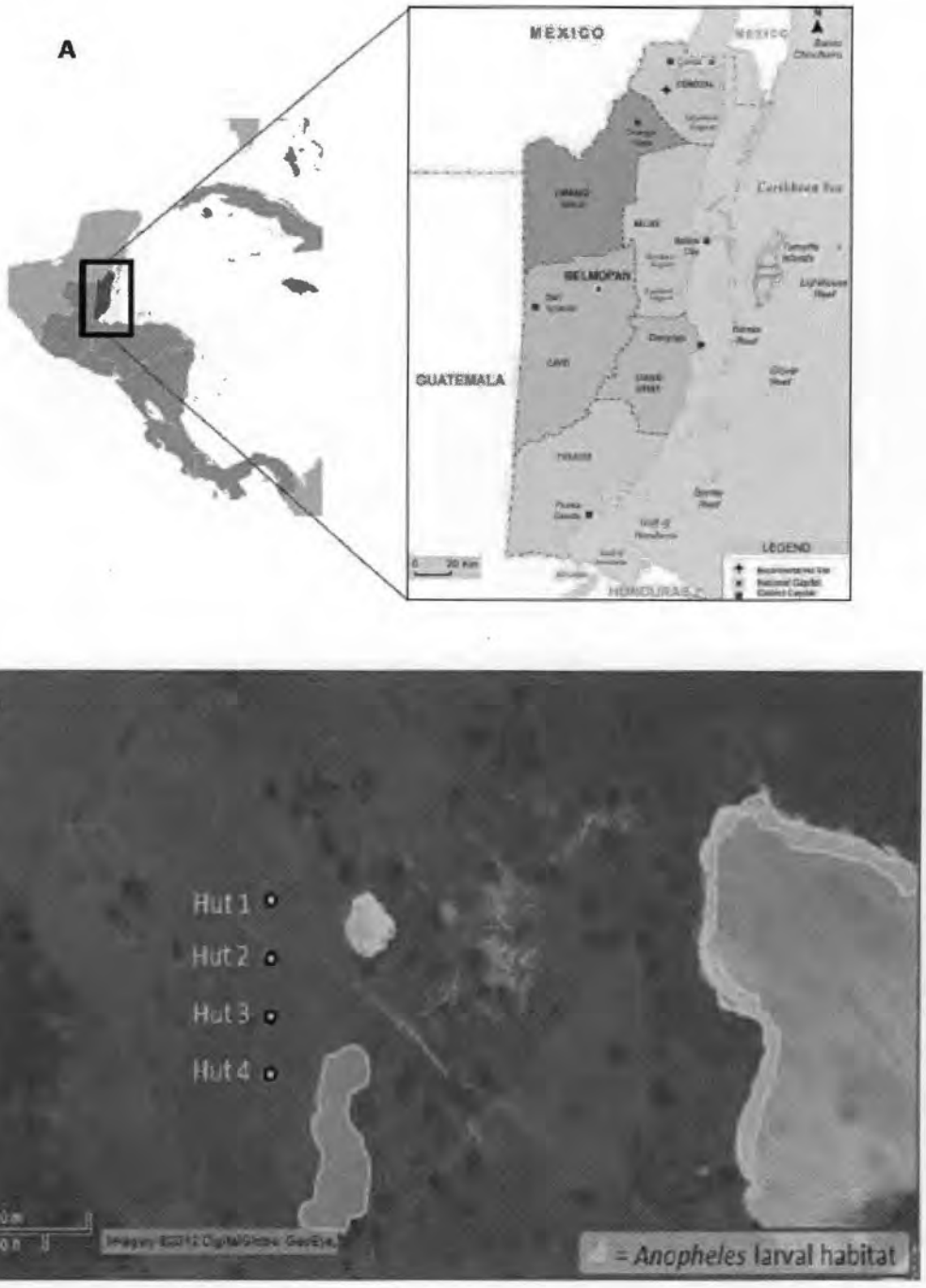


Figure 8. The Progresso Hut Site.
(A) The study site location in Corozal District, Belize. (B) Hut placement on-site, with known *Anopheles* spp. larval habitats highlighted.

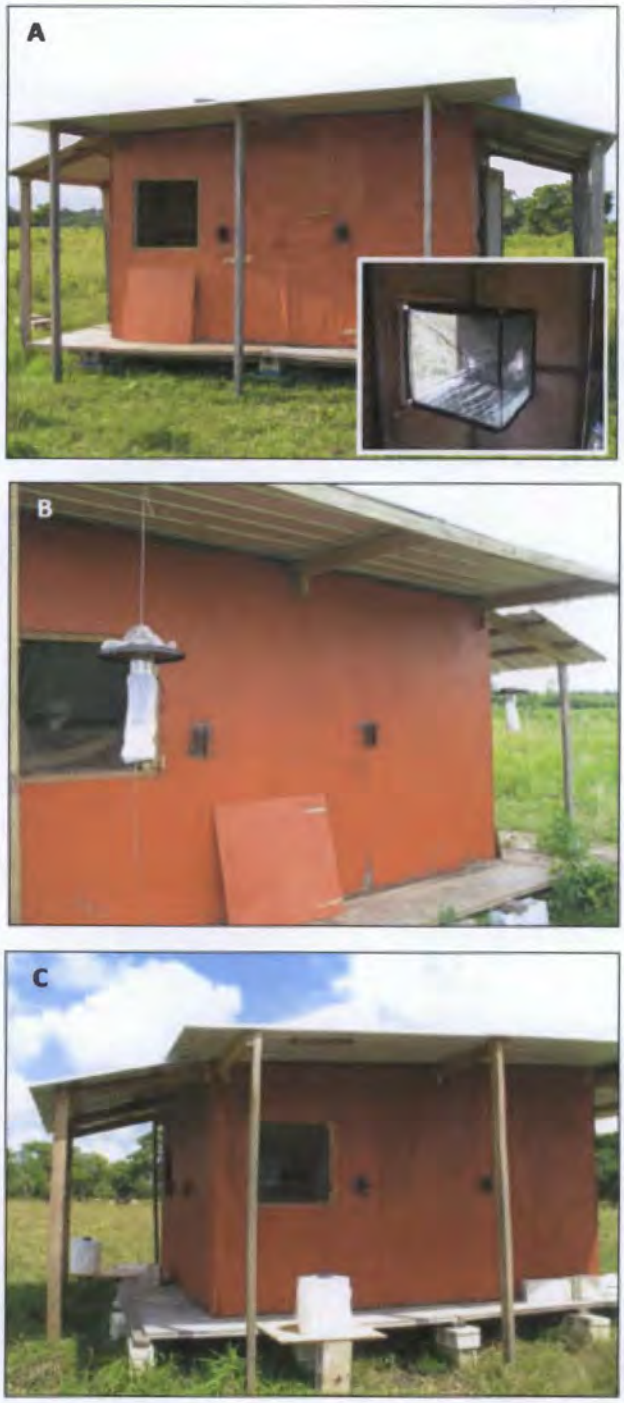


Figure 9. Experimental hut setup.
(A) An experimental hut at the field site with an open window (portal for mosquito entry), with a window interception trap used to monitor mosquito entry (inset). (B) Baited CDC Light Traps and (C) Baited BG-Sentinel™ traps positioned outside each portal of entry.

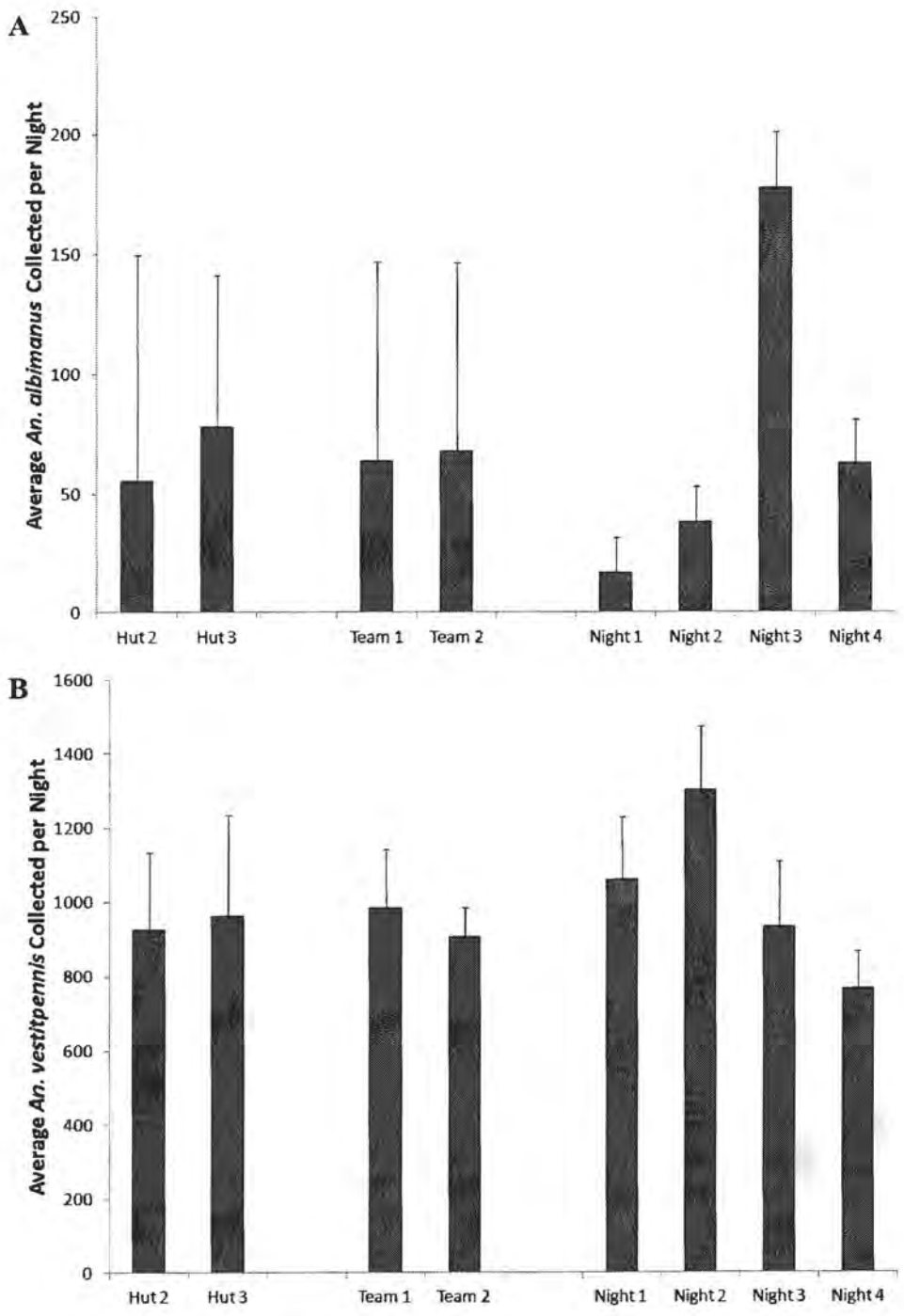


Figure 10. Baseline hut comparability, 2011.
Comparability in the numbers of mosquitoes collected for both (A)*An. albimanus* and (B) *An. vestitipennis*. ANOVA (α=0.05) showed no significant effect of hut location, collection team, or collection night.

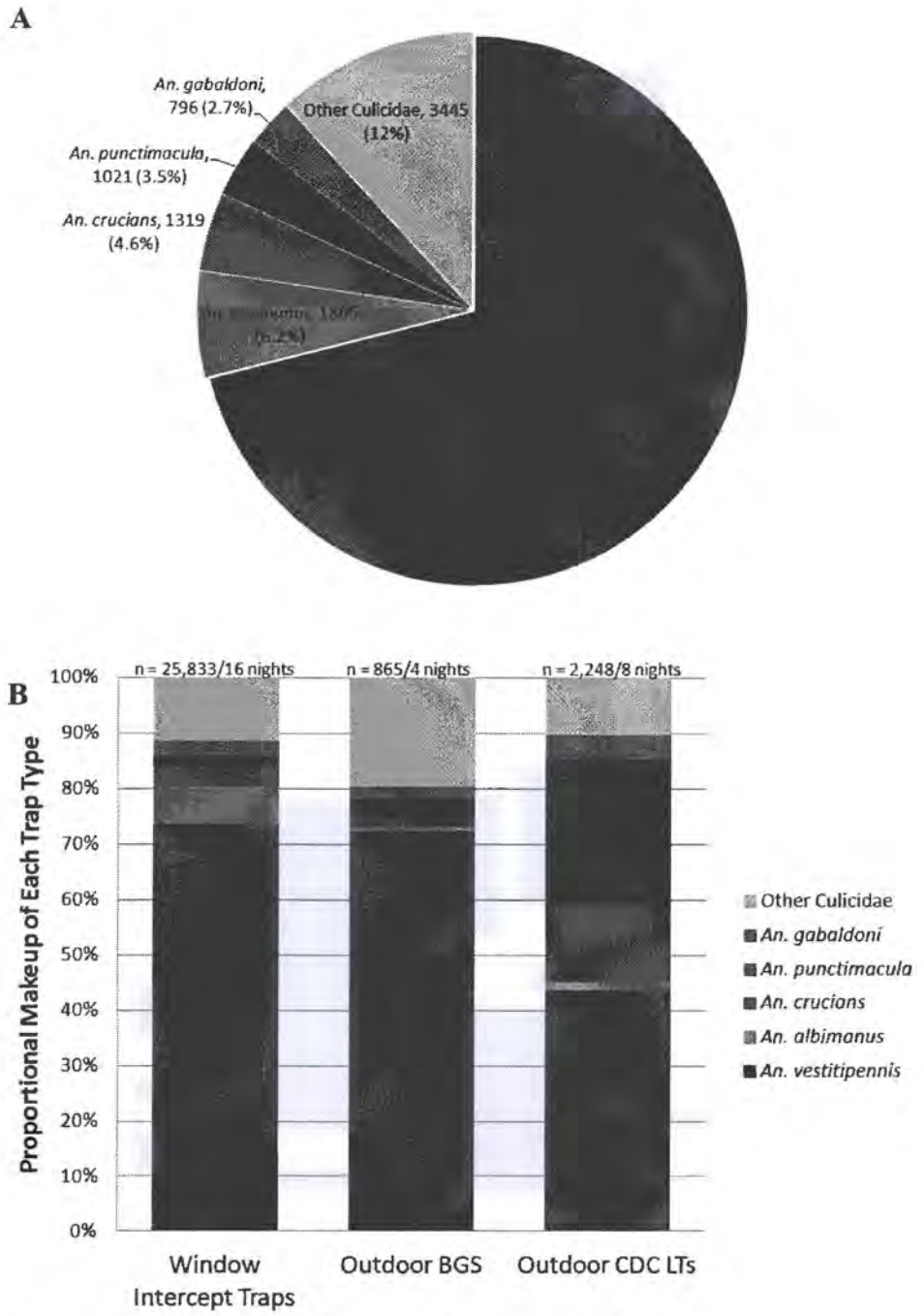


Figure 11. Mosquitoes at the Progresso Hut Site, 2011.
(A) The total adult female mosquito composition at the field site during 16 overnight (12h) collections from September to December, 2011. A total of 28,946 specimens were collected by window interception traps, baited CDC light traps (CDC LTs) and baited BG Sentinel™ traps (BGS). (B) The species composition, by proportion, of the mosquitoes collected using the three methods.

Table 2. Nightly average mosquitoes collected during four overnight (12h) collections comparing the efficacy of CDC Light Traps and BG Sentinel™ Traps[1].

	Mean (SE) Window Intercept Traps: CDC LT[2] Hut	Mean (SE) Window Intercept Traps: BGS[3] Hut	Mean (SE) Outdoor CDC LT	Mean (SE) Outdoor BGS
An. vestitipennis	1138 (102)	1160 (105)	134 (46)	130 (25)
An. albimanus	71 (11)	75 (9)	5 (2)*	1 (0)*
An. crucians	38 (7)	39 (10)	44 (41)*	4 (1)*
An. punctimacula	21 (5)	40 (14)	77 (80)*	7 (1)*
An. gabaldoni	45 (8)	57 (15)	10 (14)	3 (3)
Other Culicidae	112 (15)	165 (5)	38 (7)	38 (5)

[1]Outdoor traps were hung near the windows of experimental huts, which were fitted with interception traps and occupied by 2 collectors. Geometric means are presented, SE = standard error of the mean

[2]CDC LT = CDC miniature light trap [3]BGS = BioGents Sentinel™ Trap

* = significant difference between trap type, Student's t-test $P<0.05$

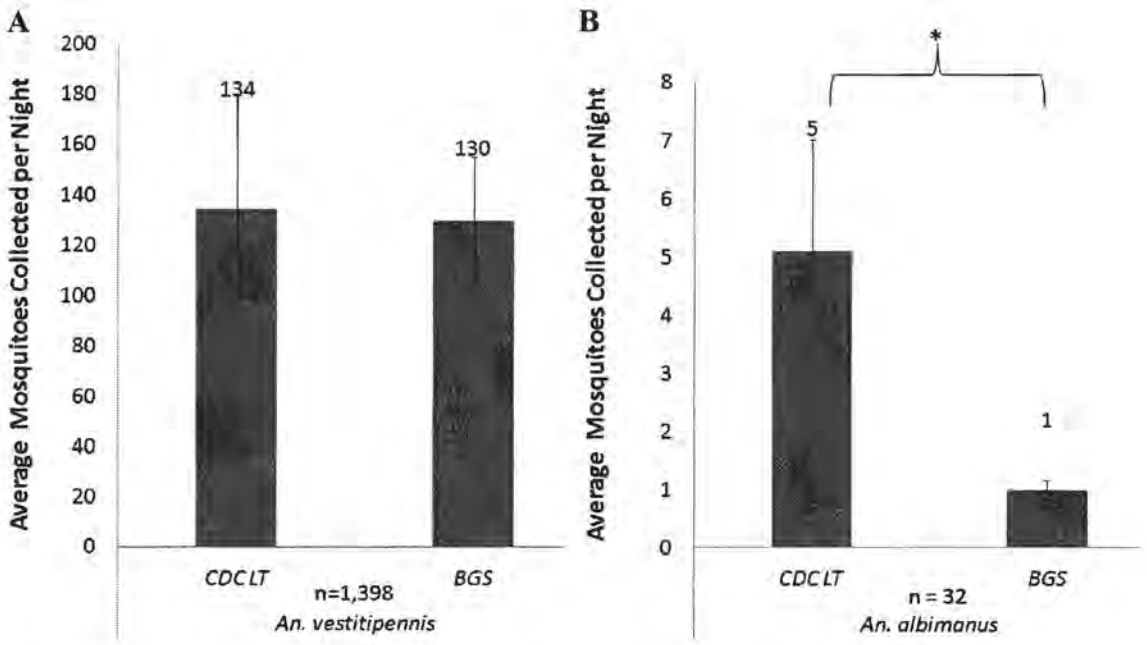


Figure 12. Comparison of two outdoor mosquito trap types.
Average number of (A) *Anopheles vestitipennis* and (B) *An. albimanus* collected in CDC light traps (CDC LTs) baited with human foot odor and in BioGents BG-Sentinel™ (BGS) traps baited with the BG-Lure™ over four all-night (12h) collections in northern Belize, Central America. Geometric means with standard error of the means are presented, * indicates a significant difference at $P<0.05$.

Table 3. Efficacy of CDC Light Traps baited with either human foot odor (sock lure) or BG-Lure™ over four all-night (12h) collections.

	Sock Lure		BG-Lure™	
	Total Collected	Nightly Mean (SE)	Total Collected	Nightly Mean (SE)
An. vestitipennis	18	2.6(2.4)*	0	0*
An. albimanus	0	0	0	0

Geometric means are presented, SE = standard error of the mean.

* = significant difference, Student's t-test $P<0.05$

Table 4. The impact of outdoor baited CDC Light Traps on mosquito entry into an experimental hut during four all-night (12h) collections[1].

	Hourly Mean (SE) Window Intercept Traps: Control[2] Hut	Hourly Mean (SE) Window Intercept Traps: CDC LT[3] Hut	Hourly Mean (SE) Outdoor CDC LT
An. vestitipennis	17.2 (1.4)	17.1 (1.3)	7.6 (0.5)
An. albimanus	3.5 (0.5)*	1.9 (0.2)*	1.1 (0.2)
An. crucians	1.0 (0.2)	1.4 (0.2)	7.5 (0.8)
An. punctimacula	0.33 (0.3)*	0.17 (0.0)*	3.9 (0.5)
An. gabaldoni	0.44 (0.1)	0.55 (0.1)	1.0 (0.2)
Other Culicidae	1.8 (0.4)	1.4 (0.3)	3.8 (0.7)

[1]Outdoor traps were hung near the windows of experimental huts, which were fitted with window interception traps and occupied by two collectors. Geometric means are presented, SE = standard error of the mean

[2]Paired control hut with no outdoor traps [3]CDC LT = CDC minature light trap

* = significant difference in hut entry, Student's t-test $P<0.05$

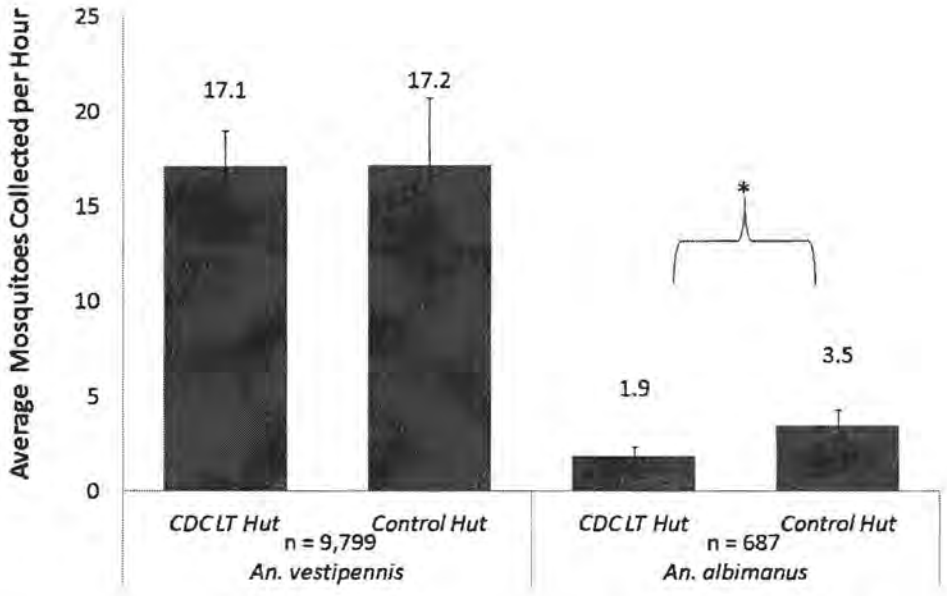


Figure 13. Effect of outdoor CDC light traps on total mosquito entry.
Average number of target vector species collected entering experimental huts with baited CDC light traps (LTs) hung outside hut windows over four all-night (12h) collections. Geometric means with standard error of the means are presented, * indicates a significant difference (Student's t-test) at $P<0.05$.

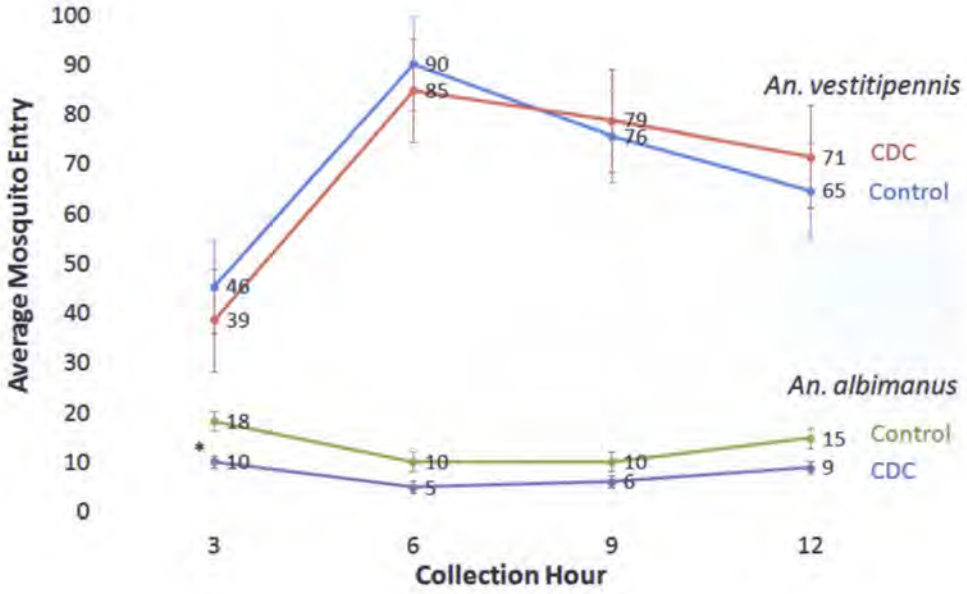


Figure 14. Impact of outdoor CDC light traps on mosquito entry temporal patterns. Entry patterns, by nightly averages aggregated into three hour blocks, of target vectors into experimental huts in the presence (CDC) and absence (Control) of outdoor CDC LTs baited with human foot odor. * Indicates a significant difference at $P<0.05$. Error bars indicate the standard error of the mean.

Table 5. Summary of meteorological conditions during all-night mosquito collections, September to December, 2011.

Evaluation	Collection Date	Wind Direction	Max Wind Speed	Min Wind Speed	Total Rain	Outdoor Mean Temp	Outdoor Max Temp	Outdoor Min Temp	Indoor Mean Temp	Indoor Max Temp	Indoor Min Temp
Baseline	28-Sep-11	SE	2.1	0	0.02	76.1	86.6	73.2	80.7	87.3	77.3
	30-Sep-11	E	1.9	0	0	73.4	80.8	70.4	75.8	80.1	73.2
	3-Oct-11	NE	4	0	0.04	73.8	85.1	71.1	77.2	85.1	73.8
	6-Oct-11	NE	1.9	0	0.02	79.3	85.1	76.7	81.8	84.4	79.4
CDC vs BG	17-Oct-11	NE	3	0	0	75.6	83	73.2	78.2	85.8	75.2
	20-Oct-11	N	1	0	0	67.8	78.7	67.8	71.3	78	65
	24-Oct-11	NW	2	0	0	67	74.5	63.5	70.1	80.1	66.3
	27-Oct-11	NE	2	0	0	73.9	80.1	69.7	77.1	84.4	73.5
CDC vs Control	3-Nov-11	NE	1	0	0	72.3	79.4	69	75.6	82.2	71.8
	10-Nov-11	NE	1	0	0	68.9	76.6	65.6	72.2	81.5	68.3
	21-Nov-11	E	1	0	0	72.4	80.8	67.7	75.1	82.2	70.4
	19-Dec-11	NE	1	0	0	69.5	75.9	64.9	73.2	78	69

CHAPTER 3: The Field Evaluation of a Push-Pull Strategy to Control Malaria Vectors in Northern Belize, Central America[3]

ABSTRACT

Campaigns for the continued reduction and eventual elimination of malaria will require new and innovative vector control tools. One novel approach being considered uses a push-pull strategy, whereby spatial repellents are used in combination with outdoor baited traps. The desired effect is the behavioral manipulation of mosquito populations to elicit movement of vectors away from people and into traps. Here, a field based Latin square experimental hut methodology was used to evaluate a prototype push-pull intervention against two target vector populations, *Anopheles albimanus* and *An. vestitipennis*, in Belize, Central America. For *An. vestitipennis*, the combined use of an indoor repellent and outdoor baited traps reduced mosquito hut entry by 39% (±1.9%) as compared to control and increased the densities of *An. vestitipennis* captured in outdoor baited traps by 48% (±26%), as compared to when no repellent was used. Against *An. albimanus*, the combined push-pull treatment similarly reduced hut entry by 54% (±14%) as compared to control; however, the presence of a repellent indoors did not affect overall outdoor trap catch densities for this species. Against both anopheline species, the combined intervention did not further reduce mosquito hut entry compared to the use of repellents alone. The prototype intervention evaluated here clearly demonstrated that

[3] The majority of Chapter 3 has been submitted to the journal PLoS ONE as:
Wagman J, Grieco J, Bautista K, Polanco J, Briceño I, King R and Achee N. In review. The Field Evaluation of a Push-Pull Strategy to Control Malaria Vectors in Northern Belize, Central America. *PLoS ONE* submission # PONE-D-14-18319
JW assumes sole responsibility for the work presented here. JW designed the study, performed the experiments, interpreted the data and wrote the manuscript. JG and NA contributed to the design of the study, interpretation of the data, and revision of the manuscript. KB, JP, IB, and RK contributed to acquisition of the data and revision of the manuscript.

push-pull strategies have potential to reduce human-vector interactions inside homes by reducing mosquito entry, and highlighted the possibility for the strategy to simultaneously decrease human-vector interactions outside of homes by increasing baited trap efficacy. However, the variation in effect on different target vectors highlights the need to characterize the underlying behavioral ecology of mosquitoes to drive optimization of intervention efficacy.

INTRODUCTION

Recent achievements in decreasing the global burden of human malaria have come about through the implementation of well-coordinated, multi-faceted and evidence-based control programs of which vector control has been an integral component (47; 67; 84; 91; 99; 149; 169). Indeed, vector control is widely recognized as an essential part of any viable plan to further control, eliminate and eradicate malaria (47; 85; 169). However, current adult vector control tools such as indoor residual spraying (IRS) and long-lasting insecticide nets (LLINs) are becoming increasingly inadequate to control disease for a variety of reasons, among which are the emergence of insecticide resistance, vector behaviors (e.g. daytime or outdoor-biting) that result in reduced intervention efficacy, and local shifts in vector species composition (13; 14; 47; 63; 97). These inadequacies, coupled with renewed calls for the global elimination and eradication of malaria in all of its complex transmission settings, underscore the critical need for novel approaches for vector control (148; 149; 167).

One novel strategy currently being developed utilizes a push-pull approach, which seeks to exploit the complementary effects of spatial repellents and mosquito traps used in combination, to decrease the probability of human-vector interactions (38; 82; 111).

Developed initially as a way to control agricultural and urban pests, push-pull interventions work by combining the repellency action of one component and the attractiveness of another in order to elicit the movement of pests away from a protected resource and towards a trap for subsequent removal from the environment (38; 114; 117). Accordingly, push-pull strategies for the control of mosquito vectors of human disease would use repellents to deter host-seeking mosquitoes from treated spaces (the 'push') and towards a baited trap (the 'pull'), which would result in their capture and removal from the peridomestic environment and thereby decrease population densities for added protection in the outdoor environment (38; 82; 111).

Although still in the proof-of-concept phase, it is easy to appreciate that the dynamics of such a strategy are complex and likely to vary according to local transmission ecologies. Nonetheless, preliminary work has been encouraging. For example, Kitau et al (2010) showed in a semi-field environment that the combined use of personal repellents (topically applied) and mosquito traps could reduce the biting rates of laboratory reared *Anopheles gambiae* more than the use of traps alone (82). Additionally, a number of researchers (7; 132; 146) have made progress towards defining the parameters of a push-pull intervention for the control of the dengue vector *Aedes aegypti*, including studies demonstrating local interest and community buy-in for the concept in both Latin America and South East Asia (111). In order to assess the potential role for push-pull in the prevention of malaria, the present study measured the impact of a prototype push-pull intervention on natural populations of two regionally important malaria vectors in Belize, Central America: *An. albimanus* and *An. vestitipennis* (12; 64). A field based, matched-control experimental hut study design was used to measure and

compare two important endpoints, 1) the reduction of host-seeking mosquito entry into the huts and 2) the efficacy of outdoor baited light traps.

MATERIALS AND METHODS

Ethics Statement

Permits and approval for this study were obtained from the Ministry of Health, Belize and the Pesticides Control Board, Belize. No protected species were sampled during these studies.

Study Site and Design

The study site, previously described in Chapter 2, was established in an open pasture surrounded by freshwater lagoons and seasonal swampland near the village of Progresso in Corozal District, Belize (N18°11'52" W88°26'6") (Fig. 15A). A Latin square study design was used to compare mosquito entry into experimental huts and outdoor traps across four different experimental conditions: 1) Control, with no interventions; 2) 'pull,' utilizing only outdoor traps; 3) 'push,' utilizing only an indoor spatial repellent; and 4) 'push-pull,' utilizing both interventions simultaneously. Experimental treatments and collection teams were rotated through each of the four huts for a total replication of 16 nights. Experiments were carried out during the rainy season of 2012, May to December, when the region experienced an average of 160 mm/month of precipitation (range 50 mm/month to 305 mm/month)(58). Temperature and humidity inside the huts were measured using HOBO® Pro Series Weatherproof Data Loggers (Forestry Suppliers Inc., Jackson, MS). Wind speed, relative humidity, temperature, and precipitation were recorded outdoors with a Davis Vantage Vue® wireless weather station (Davis Instruments, Vernon Hills, IL).

Experimental Huts and Interception Traps

Four identical experimental huts were constructed on site, approximately 50 meters apart along a straight, north-south transect (Fig. 15B). Based on previously described methodologies (9), huts were built using locally acquired materials and in a style typical of homes in rural Belize. Briefly, each structure measured 3.6 square meters and had an average roof height of 2.36 meters, creating an internal volume of roughly 30.6 m^3. Huts were raised 30 cm from the ground, resting on a cinderblock and pine wood platform. Walls and floors were constructed of an untreated pine lumber frame with plywood panels. Roofs were fashioned out of corrugated tin. Each hut had one door (182 cm X 76.2 cm), cut into the eastern facing wall, and three windows (76.2 cm^2), one in each of the remaining walls. Windows were built to accommodate interception traps for capturing mosquitoes entering the hut (Fig. 16). Based on the designs of Muirhead-Thomson (1950) and Grieco and others (2000) (61; 95), interception traps measured 76.2 cm^3 and were made of a steel frame (3.2 mm diameter rebar) covered with a green polyester netting (BioQuip Products Inc., Rancho Dominguez, CA) bag. A beveled opening prevented trapped mosquitoes from escaping, while a 20 cm diameter portal enabled the aspiration of trapped specimens from inside the hut. White polyethylene tarps (A&R Enterprises, LTD, Belize City, BZ) were installed on hut floors and baseboards to facilitate the monitoring of knocked down mosquitoes. To control for residual chemical contamination from repellent treatments, all huts and interception traps were cleaned after every four collections, prior to the rotation of treatments among huts. Hut surfaces and trap netting were sprayed and washed with a 10% bleach solution and windows and doors were left open for 24 hours.

Outdoor Baited Traps

Previous trials at the field site (Chapter 2, Fig. 12) indicated that CDC Miniature Light Traps (CDC LT) (John W. Hock Company, Gainesville, FL), baited with human foot emanations collected on cotton socks (101; 133), were the most efficacious outdoor traps for *An. vestitipennis* and *An. albimanus*. Prior to use as a mosquito lure, all socks were worn for 12h by the same individual and were utilized for a maximum of 72h after initial collection. During use, socks were placed on top of the CDC LT rain guard, and when not in use, were stored away from sunlight in sealed plastic bags at ambient temperature. During the collections, baited CDC LTs were hung outside the huts, 2m above the ground and 1m from each open window (Fig. 17B), and operated according to manufacturer's recommendations. Traps were baited, positioned and turned on 30min before sunset (~1730h) and operated until shortly after sunrise (~600h). During each 12h replicate, collection bags were replaced and processed every 2hrs.

Spatial Repellent

Transfluthrin (S.C. Johnson and Son, Inc., Racine WI) (2,3,4,6-tetrafluorobenzyl (1R)-trans-3-(2,2-dichlorovinyl)-2,2-dimethyl cyclopropanecarboxylate), a volatile synthetic pyrethroid with spatial repellent (SR) efficacy against several classes of arthropod pests including anopheline mosquitoes (48; 107; 110), was selected as the chemical repellent. Following industry guidelines for a recommended dosage of 30mg active ingredient per 9.3m^2 area (M.C. Meier, personal communication, 16 August 2011) each experimental hut (13.4m^2 floor space) received a total of 43.2mg of transfluthrin emanating passively from two 55.6cm^2 strips of nylon organdy cloth (G-Street Fabrics, Bethesda MD). Each cloth strip was treated with 21.6 mg of technical grade transfluthrin

diluted in 1mL of acetone (Ace Hardware Corp., Oak Brook, Illinois) applied evenly and allowed to air dry for 15 min following previously described methodologies (13; 65; 87). Control treatments consisted of nylon strips treated with acetone only. Transfluthrin solution was prepared and nets treated at 1200h in advance of each collection night. Nets were sealed in labeled plastic bags and kept in a light proof box at ambient temperature and humidity in preparation for transport to the field site. In the field, strips were attached to a central wood bean two meters high in the center of designated huts one hour prior to the start of mosquito collections (Fig. 17A). Here, the term spatial repellency follows the WHO 2013 definition that refers to a range of insect behaviors to include movement away from a chemical stimulus as well as interference with host detection or blood meal acquisition (172).

Mosquito Collections

At each experimental hut, mosquitoes were sampled by a two-person team during 12h overnight collections. Throughout all collection periods, the door of each hut remained closed while the open windows (with interception traps attached) provided the only entry portals for host-seeking mosquitoes. Thirty minutes before dusk (~1730h), collection teams entered each structure in order to prepare for trap processing and to establish indoor host cues. Starting at approximately 1800h and repeating every 30 minutes, one collector spent a five minute timed interval aspirating mosquitoes from each window interception trap, collecting for a total of 15 minutes. Trap openings were temporarily blocked with three inch polyurethane foam (Landy's and Sons, Ltd., Orange Walk Town, Belize) during each collection interval and were immediately re-opened after. Captured mosquitoes were stored in plastic collection cups labeled by time, hut and

unique window identifier. Each 12h collection period was divided into three hour shifts during which collectors took turns alternately processing traps and resting. All anopheline mosquitoes were identified to species using a site appropriate key (173). Ovarian dissections were conducted on a subset of target vector species (15%) to estimate age structure using parity characteristics (parous vs. nulliparous) (158).

Follow On Study

After the completion of the push-pull evaluation, an additional four-night follow-on study was performed to test if there was an observable interaction between the spatial repellent and the outdoor lure that was confounding the spatial repellency effect. A two-hut Latin square procedure was employed to evaluate the difference in window intercept catches between two experimental hut conditions: a control hut that utilized the standard push-pull intervention with indoor transfluthrin and outdoor baited CDC LTs and an experimental intervention that utilized indoor transfluthrin and outdoor, non-baited (clean socks) CDC LTs.

Data Analyses

Unless otherwise noted, Excel 2007 (Microsoft, Redmond WA) was used to $\log_e$ transform the raw numbers of mosquitoes collected and to calculate means, interval endpoints and standard errors. This data was then back-transformed, and the geometric means of mosquito densities collected are reported ± the standard error of the mean (±SE). The mean number of mosquitoes entering each hut was used to perform ANOVA using IBM SPSS Statistics v20.0 (Armonk, NY). Post-hoc analyses, including Tukey's test of Honestly Significant Differences and Wilcoxon's Signed Rank test, were used to differentiate means. Student's T-test ($\alpha = 0.05$) was also used to compare differences in

the mean number of mosquitoes trapped in CDC LTs hanging outside huts with and without repellent treatments, and to compare the mean numbers of mosquitoes collected in window intercept traps at huts utilizing outdoor CDC LTs with and without lure during the follow-on study.

RESULTS

Baseline site characterization, performed in July and August of 2012, showed *An. vestitipennis* (72%) and *An. albimanus* (7%) to represent the largest proportions of mosquito species collected (Fig. 18), as had been previously observed in 2011 (Chapter 2). Other mosquitoes encountered included *An. crucians* (5%), *An. punctimacula* (1%), *An. gabaldoni* (1%), and a number of other culicines (15%) (Fig. 18) which were not identified to species but included the following genera: *Culex*, *Psorophora*, and *Mansonia*. In addition, pre-intervention collections indicated both hut and collection team comparability (ANOVA, $\alpha = 0.05$) (Fig. 19). During treatment evaluations, a total of 21,494 mosquitoes were collected and identified, 15,411 from indoor window interception traps and 6,083 from outdoor CDC LTs (Table 6). Two of the most abundant mosquitoes collected were *An. vestitipennis* (total n=9,522) and *An. albimanus* (total n=2,933). Ambient outdoor temperatures ranged from an average nightly high of 26.9C (range 21.7C - 39.8C) to an average nightly low of 22.3C (range 16.6 – 25.1) with relative humidity averaging greater than 90% (range 57% - 100%) (Table 7). A spearman's rank correlation analysis on mosquito densities and climate variables indicated only one significant trend: a positive correlation (Spearman's rho=0.525, P=0.037) between nightly precipitation and the number of *An. albimanus* collected in window interception traps.

The number of *An. vestitipennis* and *An. albimanus* collected per night from window interception traps at each of the four experimental huts during the push-pull evaluation is shown in Table 6, with the nightly average number (geometric mean) by treatment shown in Figure 20. For each vector species, the highest mosquito densities were collected from control huts: 122 (±2.4) per night for *An. vestitipennis* and 27 (±4.1) per night for *An. albimanus*. For *An. vestitipennis* the pull treatment (CDC LTs outside windows) did not significantly reduce mosquito entry into huts, 107 (±2.5) per night, compared to the control hut, 122 (±2.4) per night (Fig. 20). Similarly, the time of peak entry was unaffected (Fig. 21). For *An. albimanus* there was a larger effect, although insignificant, on the numbers of mosquitoes entering the hut in the presence of CDC LTs, 27 (±4.1) per night, as compared to control, 18 (±3.6) per night (Fig. 20). *An. albimanus* hourly entry patterns also illustrate this reduction (Fig. 21). The use of the spatial repellent reduced nightly mosquito entry of *An. vestitipennis* by 60% (±1.7%, 95% CI) compared to the control hut (48 [±2.9] vs. 122 [±2.4]; Fig. 20). Similarly, a nightly reduction of 69% (±11%, 95% CI) was observed in *An. albimanus* entry (9 [±4.8] vs. 27 [±4.1]; Fig. 20). These reductions were significant and consistent throughout the entire collection period (Fig. 22).

The combined push-pull treatment also reduced mosquito entry compared to the control hut, but the impact was slightly less than the effect of using spatial repellent alone (Fig. 20). For *An. vestitipennis*, the push-pull reduction in mosquito entry was 39% (±1.9%, 96% CI), from 122 (±2.4) per night to 74 (±3.0) per night (Fig. 20), while for *An. albimanus* the reduction was 54% (±14%, 95% CI), from 27 (±4.1) to 13 (±5.7) (Fig. 20). The reduced repellent effect seen at the push-pull huts, compared to the push alone huts,

was not statistically significant (ANOVA, α=0.05) in terms of the absolute numbers of mosquitoes collected in the window interception traps. However, a Wilcoxon signed-rank test comparing the difference of means between the repellent alone and the combined intervention indicated that the trend may be significant: the push-pull hut collected more mosquitoes in window interception traps than the push hut on 13 of 16 night for *An. vestitipennis* (P=0.016) and on 10 of 16 nights for *An. albimanus* (P=0.038).

In the outdoor baited CDC LTs, the spatial repellent treatment increased the nightly density of *An. vestitipennis* captured by 48% (±26%, 95% CI), from 16.3 (±3.5) per night at the pull hut to 24.1 (±2.4) per night at the push-pull hut (Fig. 23); however, no effect was seen in *An. albimanus* populations (10.2 [±2.9] vs. 9.5 [±3.9] at the push-pull and control huts, respectively) (Fig. 23). Results from the follow-on study indicate that there was no effect of outdoor CDC LT bait on the spatial repellent effect of indoor transfluthrin: statistically equivalent numbers of mosquitoes were captured entering the window interception traps from each hut (Fig. 24). For *An. vestitipennis*, no differences in parity rates were observed between mosquitoes captured indoors or outdoors, or in the presence or absence of a spatial repellent, on any night (Table 8). The numbers of *An. albimanus* captured and dissected were too low for a meaningful analysis of parity trends.

DISCUSSION

This is among the first field-based evaluations of a prototype push-pull strategy to control naturally occurring vectors of human malaria. The use of multiple experimental huts allowed for the assessment of the impact of each of the components of the intervention (an indoor spatial repellent and an outdoor baited trap) separately and in combination. Additionally, the study site provided an opportunity to assess the impact of

the same experimental interventions concurrently on two anopheline species of public health importance, the more endophagic and anthropophagic *An. vestitipennis* and the more exophagic and zoophagic *An. albimanus* (37; 61; 62; 64).

Outdoor Baited Light Traps: The Pull

Data indicate that the use of baited CDC LTs had no statistically significant effect on the entry of either species into the experimental huts, although a slight decrease in entry for *An. albimanus* was noted. These results are in line with previous observations made at this study site (Chapter 2) and seem to indicate that an outdoor baited light trap, in the absence of any other mosquito control intervention, was more likely to impact the hut entry behaviors of the more naturally exophagic *An. albimanus*. Conversely, the hut entry behaviors of the endophagic vector *An. vestitipennis*, which is inherently more attracted to the internal environment of an experimental hut, were not impacted by the presence of an outdoor baited light trap.

Indoor Spatial Repellent: The Push

The impact of indoor transfluthrin emanators was clear and consistent for both species, resulting in sharp reductions in the numbers of host-seeking mosquitoes that entered window interception traps. These results are in line with previous reports of experimental hut studies from Belize that showed both *An. vestitipennis* and *An. albimanus* to be susceptible to the spatial repellent action of indoor applications of DDT (19; 61).

The combined push-pull strategy

Against *An. vestitipennis*, results show that the combined push-pull treatment simultaneously reduced mosquito entry into experimental huts, compared to control huts, and increased the efficacy of outdoor baited CDC LTs, compared to CDC LTs operating outside huts with no spatial repellent treatment. For *An. albimanus*, results indicate a similar reduction in mosquito entry associated with the push-pull treatment, but there was no comparable effect of indoor spatial repellent on increasing outdoor light trap efficacies.

The use of an indoor spatial repellent significantly and consistently reduced entry into the window interception traps for both target species, whether used alone or in conjunction with outdoor baited light traps. Considering this, along with the statistically negligible (albeit variable) effect on hut entry of outdoor light traps used alone, the data suggests that the reduced mosquito entry observed at the push-pull huts can be attributed directly to the spatial repellent activity of the transfluthrin treatment. This inference is supported by general observations that, given currently available technologies, spatial repellents can be very effective at reducing local biting pressures (8) while trapping of adults has remained largely ineffective at doing so (except when conducted on much larger scales, over longer periods of time and using more sophisticated traps than were evaluated here) (35; 71; 83; 121; 138).

Interestingly, results in the current study also showed the presence of outdoor baited CDC LTs to decrease the repellent effect of indoor transfluthrin against both target vectors. This effect remains largely unexplained, although a follow-on study comparing two huts with spatial repellent treatment and CDC LTs (one hut using baited traps and one hut using unbaited traps) indicated no negative interaction between mosquito lures

and the spatial repellent treatment, based on equivalent number of mosquitoes captured in window traps at the two huts.

Overall, the densities of *An. vestitipennis* captured in outdoor CDC LTs increased in the presence of the indoor spatial repellent as compared to when CDC LTs were used alone. Such an effect was not seen with *An. albimanus*. Reasons for this observation might again be explained based on species-specific behaviors. More endophagic species, like *An. vestitipennis*, will be more strongly attracted to the internal environment of an occupied hut during host-seeking. If the indoor environment is found to be unsuitable, it therefore may also be more likely displaced from its endophagic host seeking path into the immediate peridomestic environment, thus increasing the probability of capture by an outdoor trap positioned adjacent to the host-occupied structure. Upon detection of an unsuitable indoor environment, an exophagic species like *An. albimanus* may simply continue to search for a blood meal in a wider area outdoors, not impacting (or perhaps lowering) the probability of contact with the same outdoor traps.

Though only a subset of *An. vestitipennis* and *An. albimanus* were age graded via ovarian dissection during each collection, it is important to mention that there were no obvious differences in the crude age structures of target vector populations with regards to the location of their capture on any night. While parity rates did fluctuate temporally throughout the duration of the study, there was no evidence that any of the interventions had a differential impact on nulliparous or parous mosquitoes (Table 8).

CONCLUSION

The experiments reported here demonstrate the potential for push-pull strategies to reduce the probability of human-vector interactions both inside (by reducing mosquito

entry) and outside (by increasing the efficacy of an outdoor baited trap) of homes. However, the variation in effect seen on different target species highlights the need to identify the underlying behavioral ecology of local vectors to tailor the strategy to different transmission settings. Additionally, further elucidation of the species-specific mechanisms that drive mosquito responses to spatial repellent chemicals and baited traps is needed to properly evaluate the potential role for push-pull vector control strategies as part of any malaria prevention program.

ACKNOWLEDGEMENTS

The opinions contained herein are the private views of the authors and are not to be construed as official or reflecting the views of the Department of Defense or the Uniformed Services University of the Health Sciences. The use of commercial names does not constitute product endorsement or recommendation. We would like to thank Dr. Michael Pitts and Dr. Aisha Andrewin of the Belize Ministry of Health and Ms. Miriam Serrut of the Pesticides Control Board, Belize for their support and approval of the study. Dr. Maude Meier of S.C. Johnson and Son, Inc., Racine WI, graciously supplied the test repellent and advice on its dosage and deployment. We also thank Susana Castillo, Russell King, Ireneo Briceño, and Christopher Martinez of the Belize Vector and Ecology Center (BVEC) for their invaluable assistance in hut construction, collection and processing of mosquito samples. Particular thanks go to the rest of the collection team members and their families: Louis Balam, Ian Briceño, Ireno Briceño, Jr, Angela Caranci-Wright, Selvin García, Abner Gómez, Russell King, Jr, Adrian Tillett, German Tillett, José Tillett and Marcelo Urbina. Special acknowledgement to Mr. Albert Burns for the use of his land for the study site. Dr. Philip Coyne, Dr. Stephen Davies, Dr. D.

Scott Merrell, and Dr. Cara Olsen of the Uniformed Serviced University of the Health Sciences also provided valuable input and guidance into the experimental design and analysis.

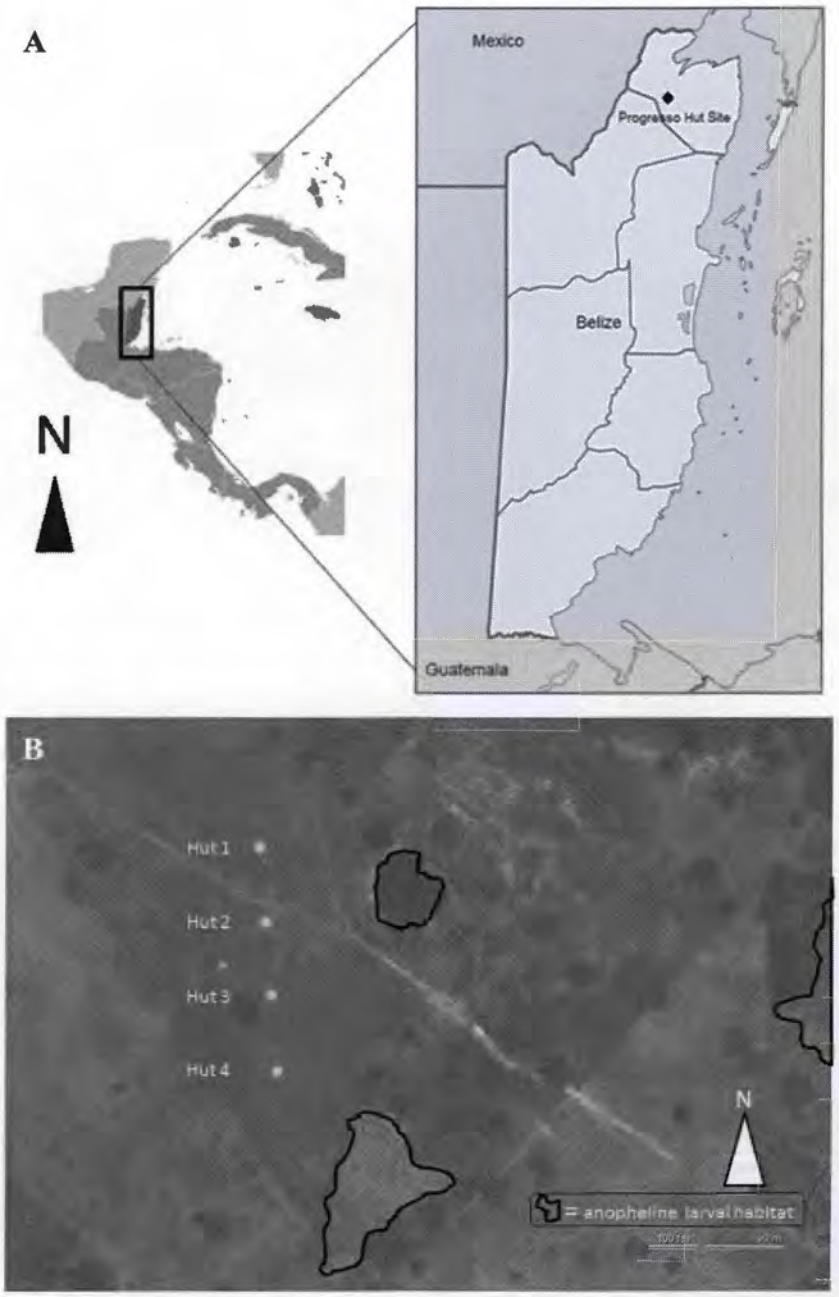


Figure 15. The experimental field site.
(A) The location of Belize in Central America, and the location of the site near Progresso Village. (B) Satellite imagery of the site showing the experimental huts and with the nearest anopheline larval habitats indicated.

Figure 16. Hut configuration.
(A) An experimental hut at the field site and (B) a window interception trap inside the same hut.

Figure 17. Experimental Treatments.
A) A spatial repellent emanator inside an experimental hut and (B) an outdoor baited CDC light trap.

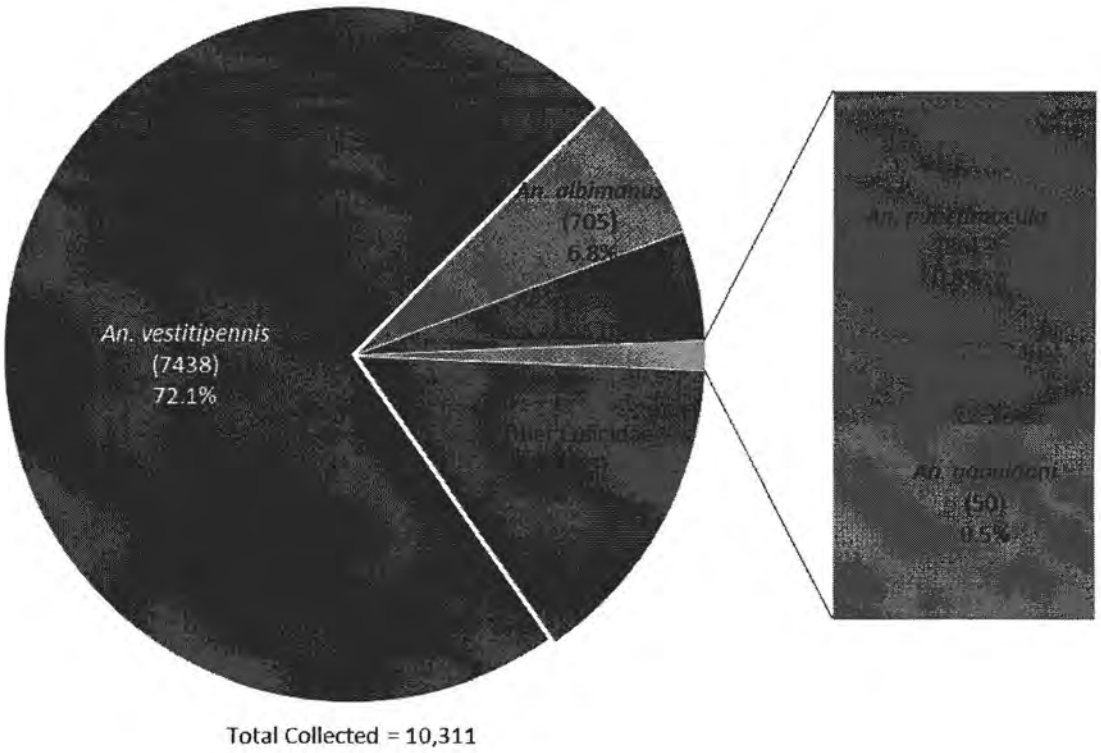


Figure 18. Adult female mosquito composition at the study site, 2012. From baseline (pre-intervention) characterization of the site from July to August, 2012.

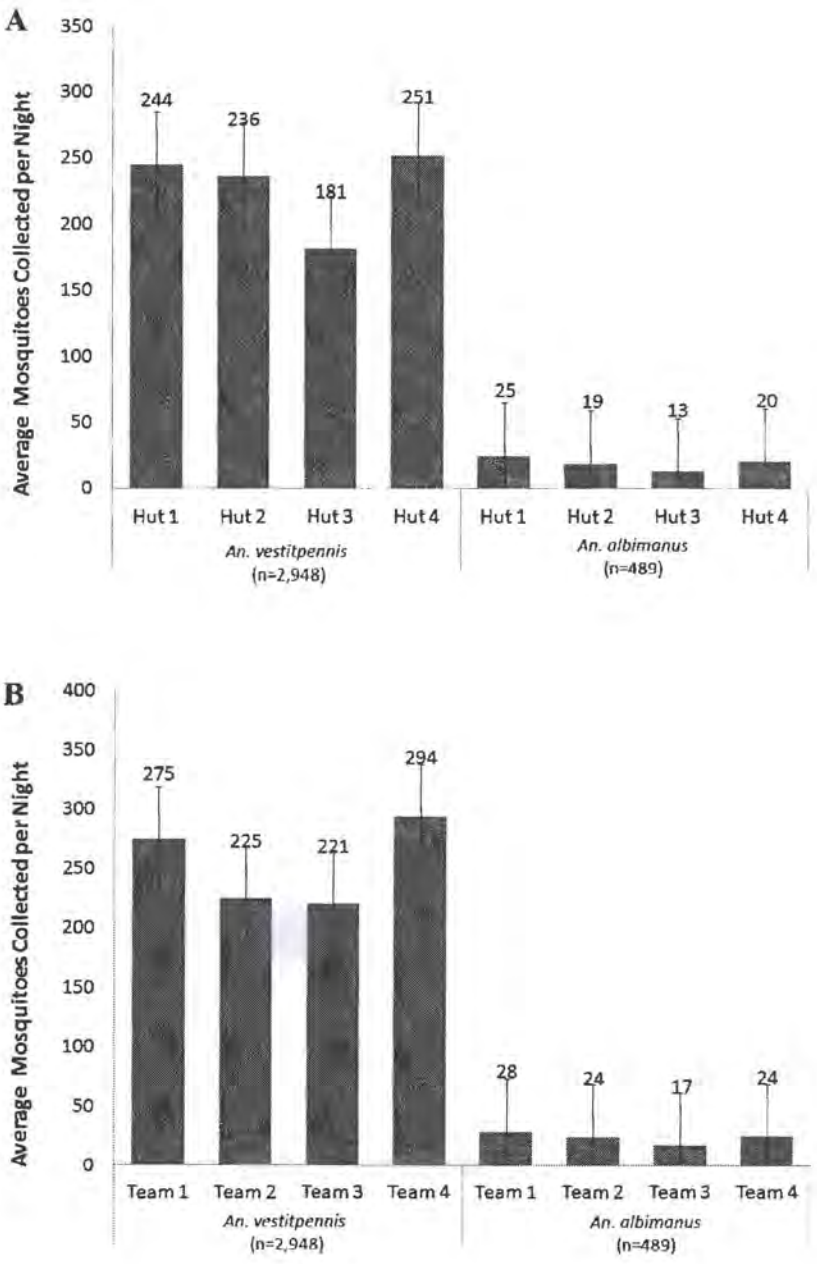


Figure 19. Baseline (pre-intervention) comparability of experimental huts, 2012. During the baseline characterization of mosquito activity, statistically equivalent numbers of target anophelines (ANOVA, $\alpha = 0.05$) were collected (A) in each hut and (B) by each collection team. Nightly (n=4) geometric means are shown; error bars represent the standard error of the mean.

Table 6. Nightly mosquito densities collected during the push-pull experimental hut evaluation.

	Indoor Interception Trap Collections								Outdoor Baited CDC LT[1] Collections			
	An. vestitipennis				*An. albimanus*				*An. vestitipennis*		*An. albimanus*	
Night	Control[2]	Pull[3]	Push[4]	P-P[5]	Control	Pull	Push	P-P	Pull	P-P	Pull	P-P
1	417	149	152	223	6	5	3	2	27	47	8	1
2	161	103	47	165	50	2	0	7	23	29	17	5
3	336	95	42	76	106	10	3	5	29	14	34	5
4	160	122	17	22	18	29	6	4	19	6	14	12
5	77	100	45	48	42	27	4	30	3	18	7	35
6	69	68	68	64	329	114	103	171	4	14	22	30
7	43	24	26	81	13	4	11	48	3	19	1	7
8	114	124	63	132	124	123	25	89	9	32	17	26
9	49	37	27	47	29	12	30	40	19	20	13	36
10	189	119	105	127	102	43	40	60	17	54	13	62
11	451	841	397	237	43	20	39	17	232	106	15	15
12	270	396	176	271	9	26	10	13	105	87	25	33
13	168	237	114	214	54	116	44	77	29	39	24	14
14	150	137	15	45	14	18	1	0	30	36	18	5
15	34	53	9	4	1	3	1	1	11	9	3	0
16	25	35	11	30	9	11	12	3	3	6	1	0
Geo Mean (SE)	122 (2.4)	107 (2.5)	48 (2.9)	74 (3.0)	27 (4.1)	18 (3.6)	9 (4.8)	13 (5.7)	16 (3.5)	24 (2.4)	10 (2.9)	10 (3.9)

[1] CDC LT = CDC Light Trap
[2] Control hut = no treatment
[3] Pull hut = outdoor CDC LT alone
[4] Push hut = indoor spatial repellent alone
[5] Push-Pull hut = combined use of outdoor CDC LT and indoor spatial repellent

Table 7. Summary of meteorological conditions during all-night mosquito collections, July to November, 2012.

Evaluation	Collection Date	Wind Direction	Average Wind Speed	Max Wind Speed	Min Wind Speed	Total Rain	OutdoorMean Temp	OutdoorMax Temp	OutdoorMin Temp	Indoor Mean Temp	Indoor Max Temp	Indoor Min Temp
Baseline	9-Jul-12	NE	NR	1.9	0	0	77.7	86.4	75.5	80.3	90.3	76.4
	12-Jul-12	NE	NR	1.9	0	0	81.4	88.6	77.7	84.7	91	79.3
	30-Jul-12	NE	NR	5	0	0.02	77.8	88.4	73	80	90.2	75.2
	13-Aug-12	NE	1.27	6	0	0	77.9	84.8	76	78.6	85.8	76.6
	23-Aug-12	NE	1.73	7	0	0.02	78.6	85.8	75.2	79.4	88.7	77
	27-Aug-12	NE	1.63	14	0	1.38	74.1	77.9	74.1	78	85.8	74.5
Push-Pull	3-Sep-12	NE	0.4	6	0	0	75.9	85.6	73.3	80.8	88	73.8
	6-Sep-12	NE	2.84	9	0	0	80	85.8	77.1	81.4	88	78
	10-Sep-12	NE	1.61	8	0	0	78.1	85.3	73.9	81.7	87.3	74.5
	13-Sep-12	NE	1.71	6	0	0	78.9	87.2	74.9	78.4	88	72.5
	17-Sep-12	SE	1.96	5	0	0	78.9	87.5	75.6	80.9	88	77.3
	24-Sep-12	NE	2.49	9	0	0	76	81.8	72.9	77.8	85.1	73.8
	27-Sep-12	NE	0.39	2	0	0.01	75.3	80	73.6	77.6	86.6	74.5
	4-Oct-12	NE	2.63	8	0	0.55	73.1	75.9	71.4	74.9	83	71.8
	8-Oct-12	NW	1.27	5	0	0	75.1	79.9	73.7	77.3	86.6	74.5
	11-Oct-12	NE	1.71	6	0	0.61	76.8	80.9	74.7	77.7	83	75.2
	14-Oct-12	NW	0.9	3	0	0	74.4	77.8	72.4	76.8	85.1	73.2
	18-Oct-12	NE	0.35	3	0	0	74	81	71.2	76	85.8	71.8
	22-Oct-12	N	1.09	4	0	0.1	74.5	75.3	73.7	75	80.8	73.2
	25-Oct-12	W	0.49	2	0	0	70.2	76.4	67.5	72.4	84.3	67.7
	22-Nov-12	NE	1.24	4	0	0	65.4	61.9	71.1	67.4	79.4	62.2
	26-Nov-12	N	1.29	5	0	0	70.35	75.3	66.1	73.6	78.7	67.7

NR = Not Recorded

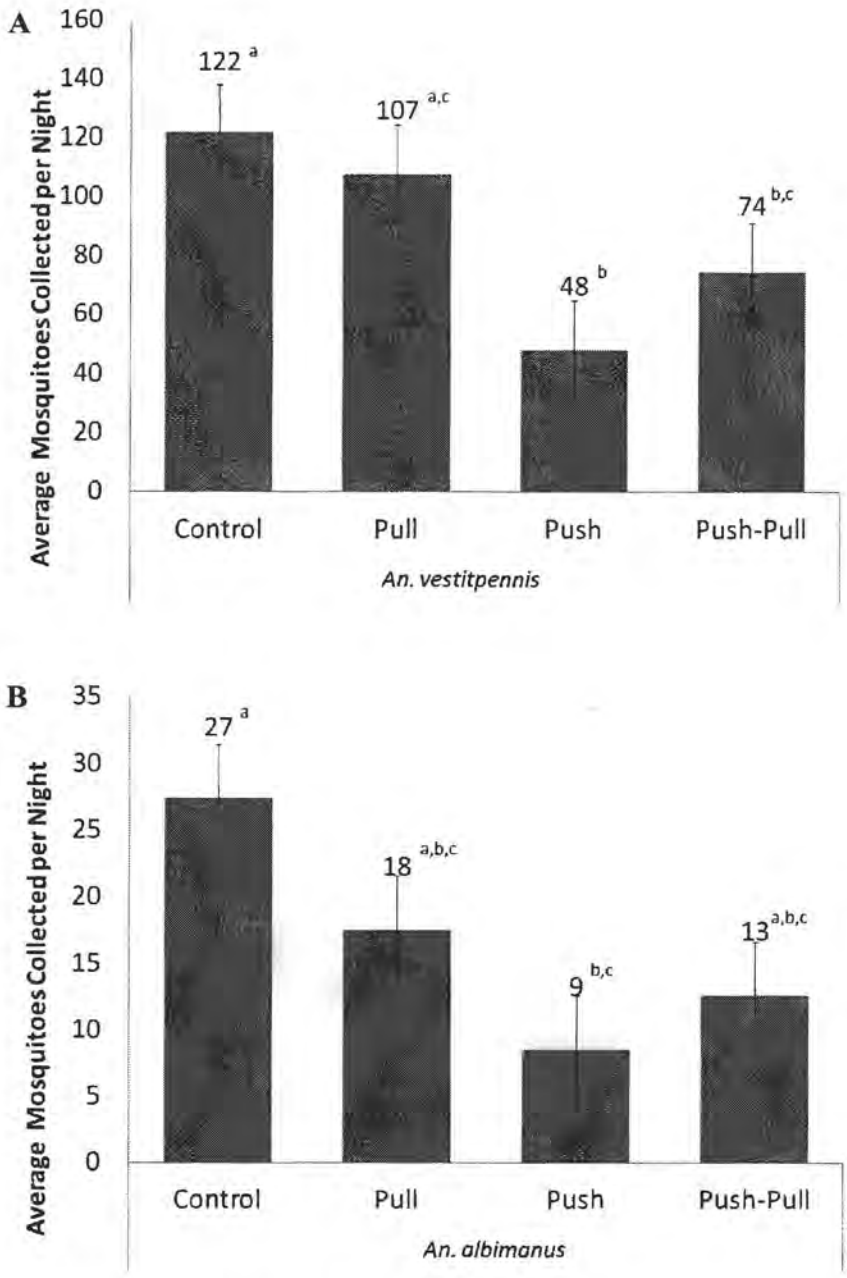


Figure 20. Reductions in mosquito entry.
The nightly averages (geometric mean, n=16) of female mosquitoes collected from window interception traps at each hut for (A) *An. vestitipennis* and (B) *An. albimanus*. Control = no intervention, Pull = outdoor baited light trap, Push = indoor spatial repellent, Push-Pull = combined intervention. Error bars show SEM, lower case letters indicate significantly different means (ANOVA with Tukey's test of honestly significant difference with α=0.05).

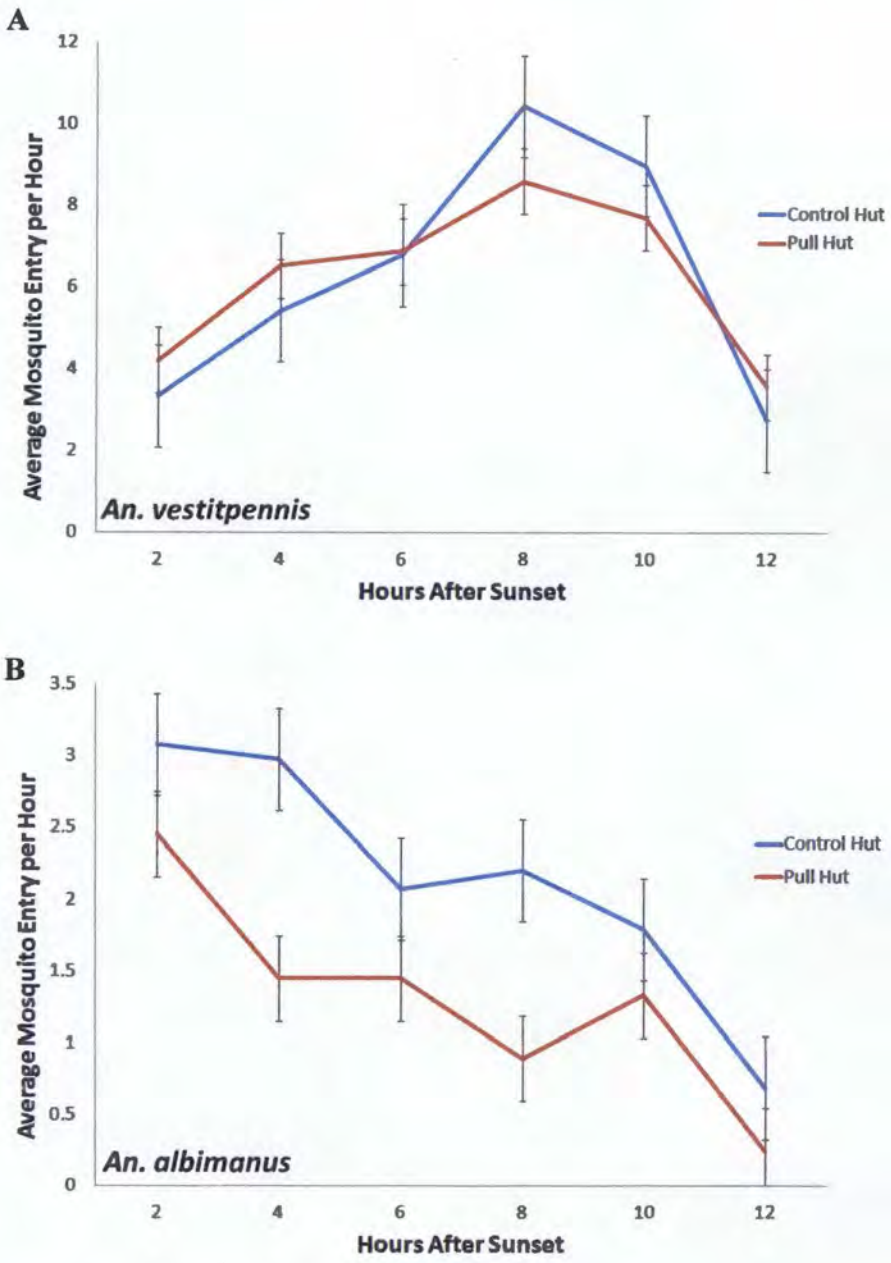


Figure 21. General patterns of mosquito hut entry.
Aggregate patterns of mosquito entry into control (no treatment) and pull (outdoor light traps) huts for (A) *Anopheles vestitipennis* and (B) *An. albimanus* throughout the study (n=16 nights). Geometric means are presented in 2h intervals, error bars represent the SEM.

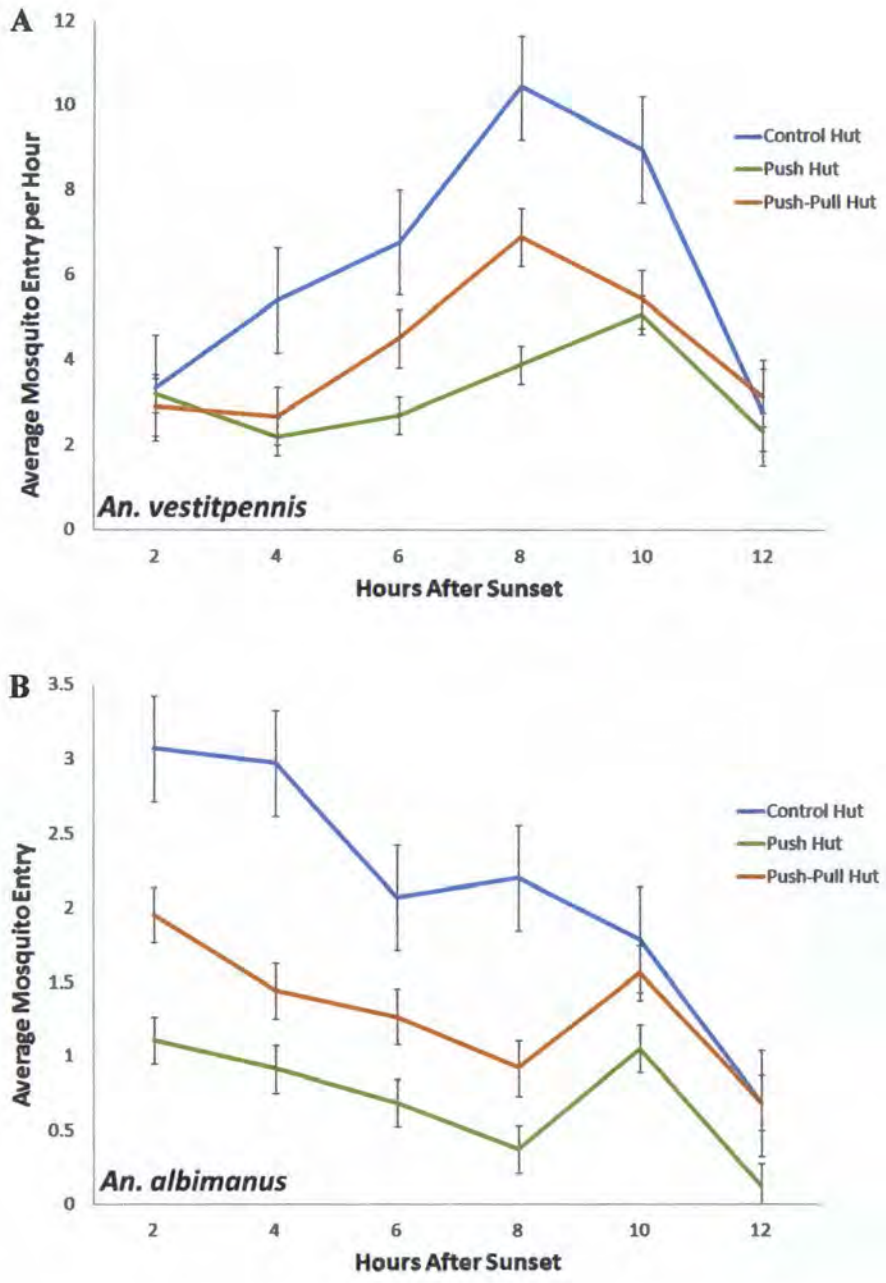


Figure 22. Effect of spatial repellent treatment on mosquito hut entry. Aggregate patterns of mosquito entry into control (no treatment), push (indoor spatial repellent only) and push-pull (indoor spatial repellent and outdoor baited traps) huts for both (A) *Anopheles vestitipennis* and (B) *An. albimanus* throughout study (n=16 nights). Geometric means are presented in 2h intervals, error bars represent the SEM.

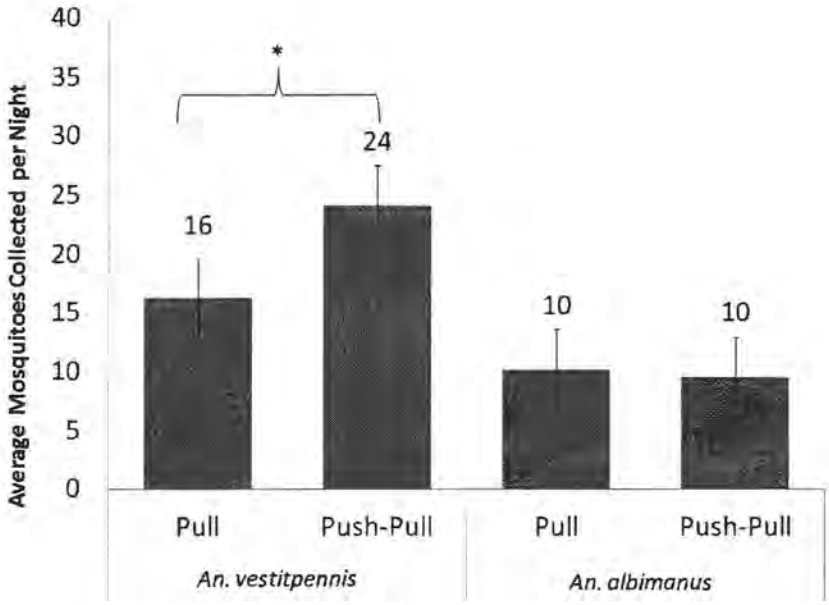


Figure 23. The impact of indoor spatial repellent use on outdoor light trap efficacy. The nightly (n=16) average numbers of mosquitoes collected from outdoor CDCLTs at the pull (outdoor light traps only) and push-pull (combined use of indoor transfluthrin and outdoor light traps) huts. Geometric means are presented, error bars show the SEM, * = statistically significant at α=0.05, Student's T-test.

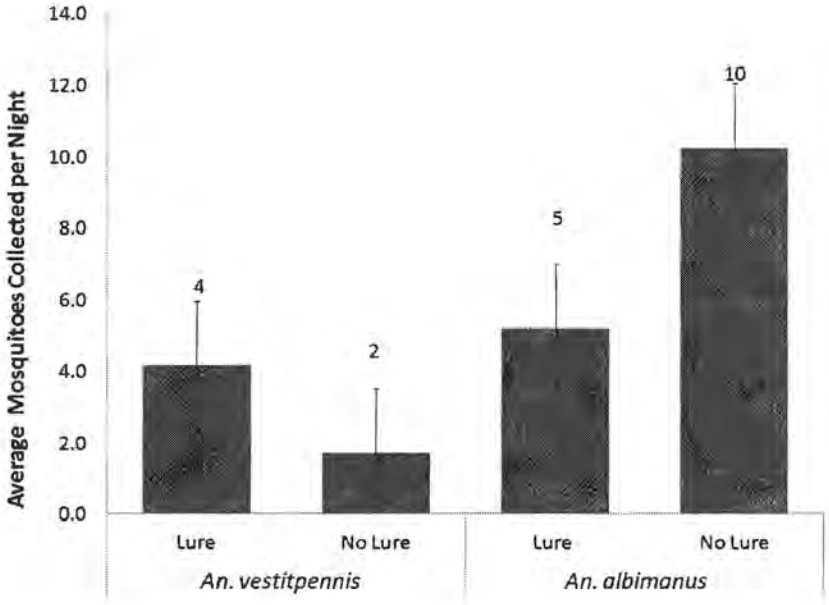


Figure 24. No interaction between outdoor mosquito lure and indoor repellent. The nightly (n=4) average numbers of mosquitoes collected from indoor window intercept traps in huts deploying baited (Lure) and unbaited (No Lure) CDCLTs. Geometric means are presented, error bars show the SEM.

Table 8. Parity rates for subset of captured *An. vestitipennis* that were age graded via ovarian dissection, by collection night[5].

	Inside[1] SR[2]		Outside[3] SR		Inside Control[4]		Outside Control	
Night	n	Parity (%)	n	Parity (%)	n	Parity (%)	n	Parity (%)
3-Sep	13	85	16	69	14	79	13	77
6-Sep	9	67	5	80	11	82	6	67
10-Sep	8	12.5	6	33	14	50	10	50
13-Sep	3	75	0	n/a	4	100	0	n/a
17-Sep	10	40	2	50	14	64.3	3	66.7
24-Sep	3	66.7	6	66.7	6	66.7	7	71.4
27-Sep	10	70	7	42.9	20	70	2	100
4-Oct	18	55.5	0	n/a	13	15.4	1	100
8-Oct	8	75	8	75	15	80	11	63
14-Oct	13	23.1	0	n/a	18	22.2	0	n/a
18-Oct	0	n/a	0	n/a	0	n/a	9	33.3
22-Oct	16	68.8	10	60	15	46.7	5	40
25-Oct	7	100	10	50	16	62.5	9	55.6
22-Nov	3	66.7	5	80	10	80	8	75
26-Nov	6	66.7	6	66.7	9	88.9	2	50

[1]Inside = collected in window intercept traps. [3]Outside = collected in light traps.
[2]SR = Experimental hut with indoor repellent. [4]Control = Experimental hut with no repellent
[5] Wilcoxon signed rank hypothesis tests showed no significant differences in parity rates among mosquitoes collected indoors vs. outdoors (P=.724) or at huts with repellent vs. without repellent (P=.131).
n/a = not applicable.

CHAPTER 4: Spatial Repellent Response to Transfluthrin in *Aedes aegypti*: Behavioral Plasticity, Heritability, and a Link Between Repellent Insensitive and Insecticide Resistant Phenotypes[4]

ABSTRACT

Background: New vector control paradigms expanding the use of spatial repellents are promising, but there are many gaps in our knowledge about how repellents work and how their long-term use might affect vector populations over time. Reported here are findings from a series of *in vitro* studies that investigated the plasticity and heritability of SR behaviors, including a link between repellent insensitivity and insecticide resistance, in *Aedes aegypti* exposed to airborne transfluthrin.

Methods: A dual-choice chamber system, consisting of a central unit connected at opposite ends to one treatment chamber containing 0.0075 nmol/cm^2 transfluthrin and one control chamber without active ingredient, was used to observe directional flight behaviors in mosquitoes exposed to passively emanating transfluthrin vapors.

Results: At baseline (F_0 generation), transfluthrin actively repelled mosquitoes in the assay system. F_0 mosquitoes repelled upon initial exposure to transfluthrin vapors were no more likely to be repelled again by subsequent exposure 24h later, but repelled mosquitoes allowed to rest for 48h were subsequently repelled at a higher proportion than was observed at baseline. Selective breeding of spatial repellent (SR) responders through 9 generations did not augment the proportions of mosquitoes repelled in any

[4] The majority of chapter 4 will be submitted to the journal Parasites and Vectors as:
Wagman J, Achee N, and Grieco J. In preparation Spatial repellency responses to transfluthrin in *Aedes aegypti*: Behavioral plasticity, heritability, and a link between repellent insensitive and insecticide resistant phenotypes
JW assumes sole responsibility for the work presented here. JW designed the study, performed the experiments, interpreted the data and wrote the manuscript. JG and NA contributed to the design of the study, interpretation of the data, and revision of the manuscript.

generation. However, selective breeding of SR non-responders did produce, after four generations, a strain of mosquitoes that was insensitive to the SR activity of transfluthrin. In CDC bottle bioassays, the SR insensitive strain also exhibited decreased sensitivity to transfluthrin toxicity, while the SR responder and unselected strains remained susceptible to the toxic effects of transfluthrin throughout the study. Cross mating F_8 SR insensitive females and wild-type F_0 males restored both repellent sensitivity and insecticide susceptibility to offspring.

Conclusions: SR responses to volatile transfluthrin are complex behaviors with multiple determinants in *Ae. aegypti*. Insensitivity to the SR action of volatile transfluthrin is a heritable, recessive trait correlated with insecticide resistance traits in *Ae. aegypti*, indicating a role for the neurotoxic irritation of mosquitoes by sub-lethal doses of airborne chemical as a mechanism by which transfluthrin can produce SR behaviors in mosquitoes. Accordingly, how prolonged exposure to sub-lethal doses of volatile pyrethroids might impact insecticide resistance in natural vector populations, and how already resistant populations might respond to a given repellent in the field, are important considerations that warrant further monitoring and study. Results also highlight the critical need to develop new active ingredients with novel mechanisms of action.

Keywords: *Aedes aegypti*, spatial repellency, transfluthrin, insecticide resistance, pyrethroid

BACKGROUND

New vector control tools and paradigms are desperately needed to complement existing approaches (148; 149; 167), and there is growing evidence to support the expanded use of spatial repellents to help address this need (6; 8; 63; 105; 122; 172). The

ultimate goal of public health interventions utilizing spatial repellents is to exploit the behavior modifying effects of certain chemicals to prevent human-vector contact and, therefore, reduce disease transmission. Such approaches are among the most promising new strategies under investigation, with much progress already shown towards defining the parameters of spatial repellent-based interventions to control the global arbovirus vector *Aedes aegypti* (7; 111; 132). However, there are gaps in our knowledge about how repellents work, including the exact molecular and physiological mechanisms by which various chemicals elicit SR behaviors in important vector species (8; 22; 45; 81; 145) and the hereditary basis by which SR behavioral traits are maintained in populations of disease vectors (143; 144).

Spatial repellency (SR) is one of several behavior modifying effects of insecticides on mosquitoes that have been recognized for decades (63; 122) and have been shown to contribute to disease reduction in many settings (8; 124). In outlining a new classification system to more accurately describe the actions of chemicals used for malaria vector control, Grieco and others (2007) defined SR actions as those that stimulate "movement away from the chemical source without the mosquito making physical contact with the treated surface" (63). An expanded concept of spatial repellency, which also includes chemical actions that interfere with host detection and/or otherwise disrupt the blood-feeding process, was established by WHO in 2013 to help determine guidelines for efficacy testing (172). Taken together, it is clear that what is casually referred to as spatial repellency is really a set of complex and multifactorial behaviors which can be generally thought of as reactions to air-borne chemical stimuli

that deter mosquitoes from entering a space to take a blood meal from an otherwise suitable host.

Despite the complexities inherent in mosquito behavioral modification, much evidence to date seems to indicate that olfactory mechanisms underlie repellent behaviors (22; 23; 79). For example, DEET, which is probably the most widely used and thoroughly studied mosquito repellent (42; 94), is thought to work either through direct olfactory stimulation (113; 145) or through interference with normal host cue detection pathways to mask the presence of a potential blood meal (44; 45). Although DEET is found in personal use products applied directly to the skin and is not, strictly speaking, a spatial repellent able to protect occupants of a defined area, knowledge of its mechanisms of action is likely to inform much of our view of how spatial repellents also function. Indeed, epidemiological and entomological evidence garnered from the use of indoor residual spraying with DDT for malaria control also supports a model whereby the SR action of the chemical results from a separate mechanism, likely olfaction, from that which produces neurotoxicity: SR activity is preserved in many locations where insecticide resistance is widely reported (125). Similar observations have also been reported in pyrethroid tolerant mosquitoes that still demonstrate behavioral avoidance of sub-lethal doses of various pyrethroids (13; 81; 87). Additionally, it has also long been observed that some proportion of mosquitoes continue to locate hosts and feed even in the presence of a repellent (53; 129), and in *Ae. aegypti* this DEET insensitivity has been shown to be a heritable trait with incomplete penetrance (144) associated with specific odorant receptor polymorphisms (44; 112).

Less clear, however, is whether or not olfactory pathways are the only physiological drivers of spatial repellent behaviors in mosquitoes. For instance, Ogoma and others have reported that airborne pyrethroids and DDT both elicit multiple behavioral effects on a given mosquito population at the same time, including repellency, irritancy, reduced blood feeding, increased 24h mortality and reduced fecundity (105). Kawada and others recently reported reduced pyrethroid (permethrin and deltamethrin) contact repellency in a strain of *Anopheles gambiae* s.s. with the L1014S *kdr* mutation, but not in strains of *An. arabiensis* or *An. funestus* s.s. with cytochrome P450 driven metabolic resistance traits, supporting a role for the non-lethal disruption of neuronal sodium ion channel function in eliciting the observed excito-repellency/irritancy behaviors (81). While they did not evaluate SR behaviors specifically, these results are in line with previous knowledge that many pyrethroid compounds (i.e., permethrin, deltamethrin and alphacypermethrin) can induce irritant and/or hyperactive responses in mosquitoes at sub-lethal concentrations (34; 86) and this hyperactivity can promote the avoidance of insecticide treated nets (134). While clear that these pyrethroid insecticides can produce contact repellency behaviors through neurologically disruptive mechanisms, whether or not a highly active and more volatile pyrethroid insecticide like transfluthrin, which also has spatial repellent properties (107), elicits the same physiological responses through airborne vapor exposures is unknown. This question is especially important as residual pyrethroids are currently the most commonly used class of public health insecticide worldwide and there are growing concerns about the rapid expansion of pyrethroid resistance in key vector species (8; 70; 116). Critically, it is unclear how the use of volatile compounds that act through the same physiological pathways as the most

commonly used residual insecticides might complicate the insecticide resistance landscape.

Given the complex and multifactorial nature of SR behaviors in mosquitoes, the molecular and hereditary drivers of the behaviors are likely to vary across different active ingredients and target organisms. Nonetheless, elucidating which mechanisms dominate in specific transmission settings is an important step to understanding how to best use spatial repellents in a public health context (106), how their long-term use might impact vector populations over time (63; 87) and could also aid in the rational design of new active ingredients (8). Here, we report on a series of *in vitro* experiments that first examined the plasticity and heritability of non-contact SR behaviors in *Ae. aegypti* that were exposed to airborne transfluthrin, and subsequently explored a link between spatial repellent insensitivity and reduced insecticide susceptibility in selectively bred strains of this important arbovirus vector.

METHODS

Test mosquitoes

Aedes aegypti (L.) mosquitoes were colonized from wild-caught P_1 larvae collected from discarded automobile tires near the Belize Vector and Ecology Center (BVEC) in Orange Walk Town, Belize (18°04.938'N, 88°33.390'W). The P_1 - F_4 generations were reared and tested at the BVEC field laboratory at ambient light, temperature and humidity. Later generations (F_5 - F_{10}) and experimental crosses were reared and tested in climate controlled conditions (28°C, 60% RH, and 12L:12D light-dark schedule) at the Uniformed Serviced University of the Health Sciences (USUHS) in Bethesda, MD. Larvae were fed Chiclid Gold fish pellets (Kyorin Co., LTD, Himeji,

Japan) and adults were provided 10% sucrose solution from soaked cotton balls *ad libitum*. F_0 adults were found to be highly susceptible to transfluthrin, malathion and DDT using standard CDC bottle assay methods (Fig. 25). Behavioral assays were performed using 5-12 day old mosquitoes, which were sorted into cohorts of 20 mosquitoes 24h prior to testing. Unmated females were sugar starved (provided only water-soaked cotton) for 24h before testing, but male mosquitoes were not starved before testing.

SR behavioral bioassay

The high throughput screening system (HITSS) SR behavioral bioassay (Fig. 26), previously described by Grieco and others (2007) (65) and recently adopted by the WHO as a standard procedure for *in vitro* efficacy testing of spatial repellents (172), was used to evaluate *Ae. aegypti* SR responses to passively emanated transfluthrin (2,3,4,6-tetrafluorobenzyl (1R)-trans-3-(2,2-dichlorovinyl)-2,2-dimethyl cyclopropanecarboxylate) (S.C. Johnson and Son, Inc., Racine WI), a volatile synthetic pyrethroid with widely demonstrated SR efficacy against mosquitoes (48; 105; 107; 110; 132). Briefly, reagent grade (unformulated) transfluthrin was dissolved in 100% acetone (Hofius Ltd./Ace Hardware, Belize City and Fisher Scientific, Waltham MA) and applied evenly by micropipette across the surface of 11cm x 25cm pieces of nylon organdy netting (No. I10N, G-Street Fabrics, Bethesda MD) and allowed to air dry a minimum of 15 minutes before use. Industry guidelines (M.C. Meier, personal communication, 16 August 2011) and concurrent experimental hut studies using transfluthrin (chapter 3) indicate a standard field application rate (FAR) of 1.35mg active ingredient per cubic meter of indoor airspace to produce SR activity against mosquitoes via passive

emanation. Accordingly, HITTS treatment nets delivering 1x the FAR into the assay system were treated with 0.9mL of a $2.2x10^{-6}$ M ($8.4x10^{-4}$ mg/mL) solution. Concentrations tested ranged from 0.5xFAR to 1000xFAR. Control nets were treated with 100% acetone only.

Bioassay test system and spatial activity index

The dual-choice chamber system, which allows the observation of directional mosquito movement in response to a single chemical stimulus outside the context of host cues, consists of a clear Plexiglas central unit connected at opposite ends to one treatment chamber housing repellent-treated netting and one control chamber housing a net treated with acetone only (Fig. 26). Cohorts of 20 mosquitoes were introduced into the central chamber and, after a 30 second acclimation period, butterfly valves situated at both ends of the central chamber were opened simultaneously to allow free movement of mosquitoes in either direction into either chamber. After a ten minute exposure period, the butterfly valves were closed and the numbers of mosquitoes in each chamber were counted. Spatial repellency is measured by considering the number of mosquitoes that have moved into the untreated, control chamber (away from the treated surface) relative to the total number of mosquitoes that have moved in either direction using a weighted spatial activity index (SAI), equal to $[(N_c- N_t)/(N_c+ N_t)]x[(N_c+ N_t)]/N]$ where N is the total number of mosquitoes per replicate and N_c and N_t are the number of mosquitoes in the control and treatment chambers, respectively. Possible values for the SAI range from 1 to -1, with a value of 1 indicating the strongest SR response possible (movement of all mosquitoes away from the chemical source), zero indicating no net response, and a value of -1 indicative of a strong attractive response (movement of all mosquitoes towards the

chemical source). To account for mosquito mortality, the total number of mosquitoes tested per each replicate was corrected using Abbott's formula.

General approach

Male and unmated female mosquitoes were tested separately and, after each experimental replicate, were identified as either SR responders (SRA^+) if they had escaped into the untreated control chamber or SR non-responders (SRA^-) if they either stayed in the central chamber or flew into the treatment chamber. Mosquitoes that were located in the treatment chamber at the end of a replicate (i.e. had made physical contact with the transfluthrin treated netting) were enumerated for statistical purposes but then discarded and not further processed or analyzed. Though both male and female mosquitoes were tested during these experiments, unless otherwise stated, only female behavior was analyzed statistically and only female results are presented here. Typically, males were tested in fewer replicates only to provide sufficient numbers of males of each behavioral phenotype (SRA^+ responders and SRA^- non-responders) for selective mating purposes.

Behavioral plasticity

To evaluate the plasticity of SR responses in control (unselected) F_0 females exposed to transfluthrin, test replicates were performed and mosquitoes were immediately collected and maintained separately based on their observed behavioral phenotype. Mosquitoes were re-assayed on a subsequent day (day 2), after either a 24h or 48h resting period, and the weighted SAI for each cohort was compared to baseline (day 1) results using Student's t-test at 95% confidence.

Heritability of SR behaviors

The heritability of SR behavioral responses was evaluated by performing test replicates and collecting mosquitoes based on their SR behavioral phenotype, as above. SR responder females were then selectively bred with SR responder males to establish an SRA^+ strain of *Ae. aegypti*, and non-responder females were bred with non-responder males to establish an SRA^- strain. Changes in the SAI scores in each strain were followed for 10 generations and were compared using ANOVA with Dunnett's test for multiple comparisons at 95% confidence. An additional control strain of *Ae. aegypti*, originating from the same field collected P_1 larvae but which was allowed to freely mate, was also maintained and tested.

Insecticide susceptibility testing

At various selection points (F_0, F_5 and F_8 generations and progeny from an experimental cross between F_9 SRA^- females and newly colonized wild type F_0 males), insecticide susceptibility testing was performed using standard CDC bottle assay methodologies (24). A diagnostic dose of $9.4x10^{-5}$ mg transfluthrin (0.125xFAR) was established using F_2 unselected control females (Table 9). Test replicates lasted one hour, with mosquito knockdown recorded every 15m and final mortality recorded at 24hr.

Statistical analysis

Unless otherwise noted, SAI scores were calculated for each mosquito strain at each time point using 180 total mosquitoes, consisting of 9 replicates of 20 mosquitoes each, following established procedures (172). Raw data was organized and descriptive analyses were performed using Excel 2007 (Microsoft Corp., Albuquerque NM). A non-parametric signed rank test (PROC UNIVARIATE) in SAS v8 statistical software (SAS

Institute Inc., Cary, NC) was used to determine if mean SAI values were different from zero for each test population. SAI values were compared between populations via Student's t-test and ANOVA with Dunnett's test for multiple comparisons using SPSS Statistics 22 software (IBM Corp., Armonk NY). All analyses was performed at α=0.05.

RESULTS

HITSS SR dose-response curve

A dose-response curve was established using unselected (control) females by varying the dose of transfluthrin in the HITSS treatment chamber and measuring differences in corresponding SAI values and overall assay mortality (Fig. 27). The dose corresponding to 1xFAR (1.35 mg/m^3) produced the largest SAI value (0.10, significantly greater than zero at P<0.02) and an overall non-contact mortality of only 2.8%, appropriate for all subsequent HITSS SR tests.

Behavioral plasticity

Two variations of the behavioral plasticity experiment were performed using F_0 mosquitoes, with differing results (Table 10; Fig. 28). During the first experiment, mosquito cohorts (total n=180 mosquitoes, average baseline SAI=0.08 ±0.09) were re-assayed after a 24 hour rest period and results indicated a large degree of plasticity in behavioral responses to the repellent: mosquitoes repelled on day one (n=29) were not more likely to be repelled again on day two (SAI=0.03 ± 0.02) (Fig. 28). Mosquitoes not repelled on day one (n=129) were equally unlikely to be repelled on day two (SAI=0.03 ±0.10) (Fig. 28). For the second experiment, mosquitoes (total n=280, average baseline SAI=0.05 ±0.07) were not re-assayed until the second day after the original test, 48 hours after initial exposure. Unlike mosquitoes that were allowed to rest for 24hr, day one

repellent responders from this cohort (n=60) were more likely to be repelled again on day two (SAI=0.30 ±0.08, $P<0.05$) (Fig. 28). As was observed in the first experiment, non-responding mosquitoes from this experiment (n=155) were also equally non-responsive on day two (SAI=0.06±0.12) (Fig. 28).

Heritability of SR behaviors

The baseline average SAI value for F_0 female mosquitoes, which gave rise to all subsequent SRA^+ and SRA^- lineages, was 0.14 ±0.18 (significantly greater than zero at $P<0.02$), confirming that parental mosquitoes were actively repelled by volatile transfluthrin in the assay system. Selective breeding experiments were then carried out through the F_9 generation (Table 11). SAI results from the unselected control strain (Fig. 29) and the SRA^+ strain (Fig.30) did not indicate any changes in behavioral responses to volatile transfluthrin at any time point compared to baseline (no significant differences at $P=0.05$). Results from the SRA^- strain, on the other hand, showed a steady decrease in SAI scores, which reached statistical significance ($P<0.05$) by the F_3 generation (SAI=-0.05 ±0.12) (Fig. 30). This SR insensitive phenotype was confirmed in each subsequent SRA^- generation, with the exception of the F_7 cohort in which the reduced SAI value (0.02 ±0.11) was not significant at $P=0.05$ (Fig. 30).

Link between the repellent insensitive and insecticide resistant phenotypes

Baseline CDC bottle tests indicated greater than 95% susceptibility to transfluthrin toxicity (24hr mortality) at the diagnostic dose in the F_0 parental mosquitoes that gave rise to all selectively bred strains (Fig. 31A). Insecticide susceptibility was then reevaluated in the F_5 and F_8 generations (Fig. 31A). For the colony (unselected control, Fig. 32) and SRA^+ (responder, Fig. 31) strains, no significant changes in insecticide

susceptibility were noted by either 24hr mortality or time to knockdown. In the selectively bred SRA$^-$ repellent insensitive strain there was a moderate but significant (P<0.05) 23% reduction in mortality observed in the F_6 generation compared to the control strain (60% ±1% vs. 95% ±6%) while the F_8 SRA$^-$ strain was highly resistant with a mortality of just 14% ±11%, a significant (P<0.01) 77% reduction in mortality compared to the unselected control strain (Fig. 31A).

Experimental cross of F_8 SRA$^-$ females and wild type F_0 males restored both SR sensitivity and insecticide susceptibility

An additional round of selective breeding of F_8 SRA$^-$ non-responders gave rise to F_9 SRA$^-$ mosquitoes that continued to exhibit repellent insensitivity (SAI=-0.04 ±0.15) (Fig. 30) as well as significantly decreased CDC bottle assay knockdown and 24h mortality (13% ±13%) (Fig. 31). Mating females from the same F_8 SRA$^-$ population with wild type F_0 males newly colonized from the same location in Belize, however, restored both transfluthrin spatial repellent sensitivity (SAI= 0.11 ±0.10)(Fig. 30) and insecticide susceptibility (24h mortality = 84% ±7%) in the resulting progeny (Fig. 31).

DISCUSSION

The *in vitro* SR behaviors observed here were relatively plastic in that individual behavioral responses observed on day one were not consistent with subsequent behaviors observed upon identical chemical exposures at a later time point, reinforcing the notion that spatial repellency is a complex behavior with multiple determinants some of which are likely non-heritable (143) . Despite the overall high degree of variability in repellent behaviors on subsequent days, however, active SR responses were clearly more reproducible in mosquitoes that were given 48hr rest compared to those given only 24hr

rest (Fig. 28). This observation is consistent with other field (132) and laboratory (69) experiments that have shown post exposure habituation of mosquito behaviors that gradually resolve after appropriate recovery periods. The specific mechanisms driving these prolonged changes in behavior and their recovery, however, remain untested and in need of further investigation.

In the second set of experiments, SR responders (SRA^+) and non-responders (SRA^-) were identified and selectively bred for 9 generations. One of the possible outcomes of these experiments was the establishment of an SRA^+ strain of *Ae. aegypti* with increased sensitivity to the SR action of volatile transfluthrin, and it was originally hypothesized that such a strain of super-responders might possess olfactory receptors with a particular affinity for detecting airborne transfluthrin. However, SR responses were not augmented in the selectively bred SRA^+ strain at any time point. Conversely, though, there was a clear reduction in SR behaviors noted in the SRA^- strain, ultimately leading to a strain of mosquitoes insensitive to the SR activity of volatile transfluthrin. These results do not preclude the possibility that transfluthrin might elicit some SR behaviors by activating and/or interrupting certain olfactory pathways. In fact, the reduction in repellent sensitivity observed in the SRA^- strain is in line with previous work by Stanczyk and others that similarly demonstrated heritability of a DEET insensitivity trait in mosquitoes - and further linked the phenomenon to changes in antennal olfactory reception (144). Though similar in outcome, the DEET insensitivity trait described by Stanczyk was clearly dominant, while the transfluthrin insensitivity observed here was restored after a single cross of SRA^- females with repellent sensitive wild type males. Additionally, the HITSS SR system used here is unique in that it is designed to permit the

observation of directional mosquito movement absent any attractive stimuli, thus allowing for the measurement of active spatial repellency as a distinct entity not confounded by attraction inhibition. Accordingly, it is likely that the transfluthrin insensitive phenotype observed here relies on a different mechanism of action than the DEET insensitive phenotypes, which have been previously linked to changes in antennae sesillum function (44; 144).

As mentioned above, many insecticidal compounds are known to induce irritant and/or hyperactive responses in mosquitoes at sub-lethal doses (34; 86; 132), and this hyperactivity has been observed to promote the avoidance of treated surfaces (134). These particular behavior modifying effects are sometimes referred to as excito-repellency, which has been defined as the action of irritating a mosquito sufficiently so that it flies away from the source of the chemical before knockdown or death occurs (42; 63). In this context, the strong correlation between reduced insecticide susceptibility in CDC bottle bioassays and SR insensitivity in HITSS bioassays observed in the selectively bred SRA^- strain suggests that the SR behaviors observed here resulted from neurotoxic irritation of mosquitoes by sub-lethal doses of airborne transfluthrin. This view is bolstered by links between insecticide resistance (particularly *kdr* mutations) and decreased excito-repellency behaviors that have been identified in some field populations of mosquitoes (81; 100).

In addition to suggesting the neuro-physiological irritation of mosquitoes by active ingredient vapors as a primary mechanism by which transfluthrin can elicit SR behaviors in *Ae. aegypti*, the results of these selective breeding experiments are also notable for having experimentally reduced insecticide susceptibility in a population of

vectors exposed only to sub-lethal doses of an airborne insecticide. This is of particular importance as one of the proposed benefits to the expanded use of spatial repellents in vector control programs is the potential to alleviate much of the selective pressure that encourages the emergence of insecticide resistance from sustained use of toxic interventions in the current vector control paradigm (8; 29; 105; 122). Our results indicate that if a repellent elicits SR behaviors in the target vector through, at least in part, the same mechanisms that produce toxicity in larger doses, then the potential for selecting resistance traits might remain. It is important to consider, however, that when populations of SR responders and non-responders were allowed to mate freely (control strains), repellent sensitivity and insecticide susceptibility were maintained. The *in vitro* selective breeding approach used here favored the emergence of repellent insensitivity/decreased insecticide susceptibility only when SR insensitive females were mated exclusively with SR insensitive males. The degree to which natural mosquito populations would experience the same selective pressure in a standalone SR-based system is uncertain. Firstly, it is difficult to imagine a scenario in which repellent insensitive or repellent sensitive individuals that survive exposure to a volatile insecticide would significantly out-compete one another post-exposure, particularly when it has been shown that the use of coils to deliver airborne pyrethroids results in the decreased fitness of all mosquitoes, even those not repelled, in terms of decreased blood meal acquisition, fecundity, and delayed (24h) toxicity (105). Additionally, it is not known how or to what degree chemical exposure to repellents might affect natural male mosquito populations in an operational setting, exposures that are likely to vary significantly according to where

the active ingredient source is placed and the typical mating behaviors of the target vector.

Nonetheless, it is essential to consider these results while recognizing that pyrethroids are the most commonly used class of insecticide worldwide (8; 47; 88). Indeed, for public health applications pyrethroid use constitutes the front line approach for both indoor residual spraying (166) and insecticide treated bed nets (164), and, unsurprisingly, significant and growing concerns over the rapid spread pyrethroid resistance are prevalent (70; 102; 116). Against this backdrop, these findings are potentially more worrisome, as the effects of introducing a volatile pyrethroid repellent in an area where residual pyrethroids are already in use are unknown and require further evaluation and monitoring. Clearly, more work must be done to define what these observations mean within the larger landscape of pyrethroid use, including how prolonged exposure to sub-lethal doses of volatile transfluthrin might impact insecticide resistance in natural vector populations and how already resistant populations might respond to a given repellent in the field. Furthermore, given the clear evidence that spatial repellent effects can produce beneficial public health outcomes (8; 63; 105; 122), these results suggest that an ideal repellent active ingredient would not only have a low toxicity profile but also be unrelated to the chemical classes currently used in vector control. Acknowledging this highlights the pressing need to identify new insect behavior modifying compounds with novel mechanisms of action (85).

CONCLUSIONS

Collectively, these results show that the *in vitro* SR responses observed are complex behaviors with a mix of heritable and non-heritable determinants. Based on the

observed link between the SR insensitive phenotype and decreased insecticide susceptibility, evidence also supports a model whereby sub-lethal doses of volatile transfluthrin can elicit spatial repellent responses in *Ae. aegypti* by inducing a hyperactive or agitated state via neurotoxic pathways, likely independent of olfactory stimulation or interruption. Care should be taken before extrapolating these results to other active ingredients or vector species, and it should be emphasized that these results do not indicate that transfluthrin elicits SR behaviors in *Ae. aegypti* exclusively by disrupting motor-neuron activity: olfactory and/or gustatory pathways may also play a role, whether via active detection and avoidance of odor cues or through the disruption of host detection and/or feeding. Additionally, the appearance of decreased insecticide susceptibility in the selectively bred offspring of mosquitoes exposed only to sub-lethal insecticide vapors raises some important questions about how the long-term use of repellents might impact vector populations over time. The answer to these questions will be dependent on several factors including which molecular mechanisms are driving specific repellent behaviors, the hereditary nature of repellent sensitivity and insensitivity, and the other physiological effects of using sub-lethal concentrations of compounds that have insecticidal, as well as repellent, properties. Though the story is complex and further research is needed to better understand all of the physiological drivers of SR behaviors, evidence still supports the expanded use of spatial repellents in public health applications to control disease vectors, albeit with continued monitoring of potential changes in target vector repellent sensitivities and/or insecticide susceptibilities and a continued emphasis on the need to develop new active ingredients with novel, non-toxic mechanisms of action.

Competing Interests

The authors declare that they have no competing interests.

Acknowledgements

Thank you to Dr. Maude Meier of S.C. Johnson and Son, Inc., Racine WI for graciously providing the test repellent. Thanks to Angela Carracci Wright, of BVEC/USUHS for helping to collect mosquitoes in 2014. Also at USUHS, Dr. Cara Olsen assisted with the statistical analyses and, along with Dr. Philip Coyne, Dr. Stephen Davies, and Dr. D. Scott Merrell provided valuable input into the experimental design and interpretation.

Endnotes

The opinions contained herein are the private views of the authors and are not to be construed as official or reflecting the views of the Department of Defense or the Uniformed Services University of the Health Sciences. Mention of specific commercial products does not constitute an endorsement or recommendation

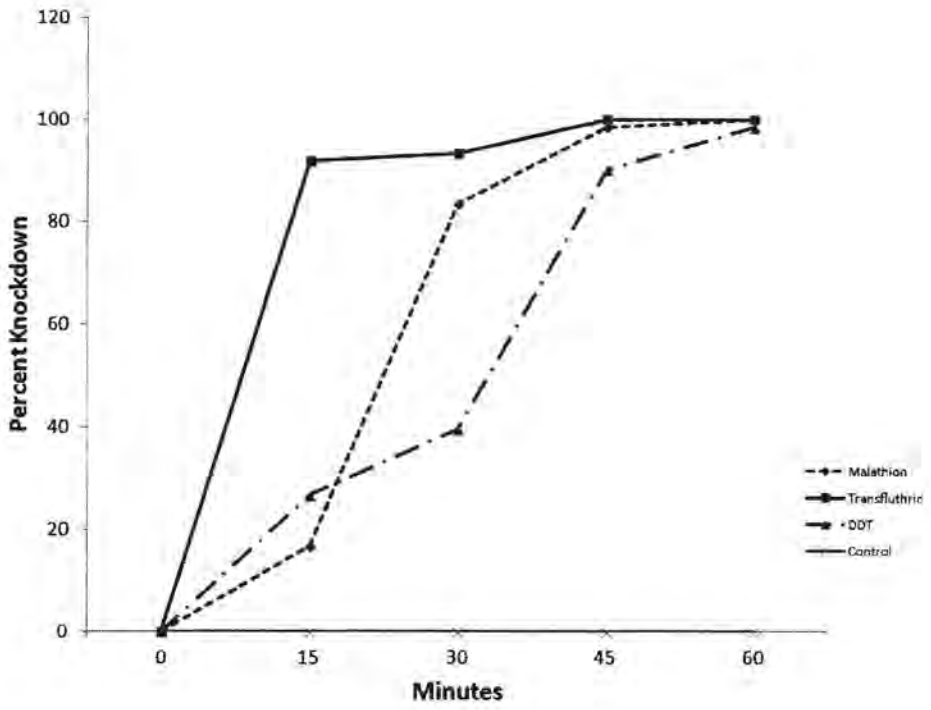


Figure 25. *Aedes aegypti* (Belize) F_0 insecticide susceptibilities.
CDC bottle assay knockdown by time. Doses were: 75 µg/bottle DDT, 50 µg/bottle malathion, and 7.5 µg/bottle transfluthrin. 24 hr mortality was greater than 95% for all chemicals tested. For DDT and malathion, these are the standard CDC bottle assay diagnostic doses (Brogdon and Chan, 2013). For transfluthrin, the dosage corresponds to 50% of the recommended standard for permethrin (CDC bottle assay standards have yet to be established for transfluthrin).

Figure 26. The high throughput screening system (HITSS) spatial repellency bioassay.
The treatment chamber (right hand metal cylinder) is covered internally by nylon organdy netting treated with transfluthrin dissolved in 100% acetone. The control chamber (left hand metal cylinder) contains netting treated with acetone only. Cohorts of 20 mosquitoes are introduced into the central (clear) chamber and directional flight behaviors are observed (Adapted from Grieco, et al. 2007., *J Am Mosq Control Assoc* 2005, 21:404-411).

Table 9. Establishment of a diagnostic dose of transfluthrin for use in CDC bottle bioassays[1].

	Knockdown				
Dose[2]	15m	30m	45m	60m	24hr Mortality
10x	100	100	100	100	100
2x	100	100	100	100	100
1x	100	100	100	100	100
0.5x	100	100	100	100	100
0.25x	100	100	100	100	100
0.125x	26.7	50.0	76.7	96.7	93.3
0.063x	3.3	3.3	60.0	86.7	90.0
0.01x	0	0	0	0	0

[1] F_2 colony (unselected control) female mosquitoes, 5-8 days old, 24h sugar starved
[2] Doses are relative to the standard field application rate (FAR), where 1xFAR = 1.35 mg/m^3 (2.0 nm per bottle).

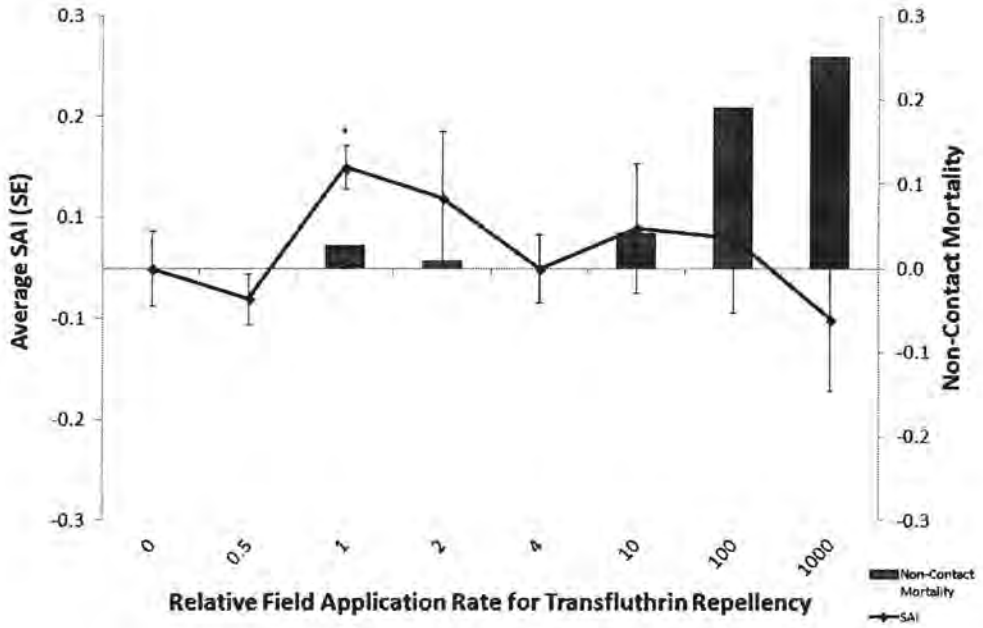


Figure 27. Spatial Repellency Dose-Response Curve.
Weighted spatial activity index (SAI) scores and non-contact mortality for unselected (control) female *Aedes aegypti* exposed to varying doses of volatile transfluthrin in the spatial repellency bioassay. Each concentration was tested with 9 replicates of 20 mosquitoes.* indicates an average SAI significantly different from zero at $P<0.05$, error bars indicate the standard error of the mean. Transfluthrin concentrations on the X-axis are shown relative to the standard field application rate (FAR), where 1xFAR = 1.35 mg/m^3.

Table 10. Plasticity of spatial repellency behaviors in *Aedes aegypti*[1] females exposed to volatile transfluthrin (1.35 mg/m^3).

	Cohort	Number of Trials (No. Mosqu.)	Mean Percent Active (SE)	Mean SAI[2]	SR[3]	*P*[4]
24h Rest	Baseline (Day 1)	9 (180)	24 (19)	0.08 (0.03)	25	0.04
	Responders (Day 2)	2 (29)	17 (4)	0.03 (0.03)	2	0.48
	Non-Responders (Day 2)	7 (129)	13 (8)	0.04 (0.05)	11	0.38
48h Rest	Baseline (Day 1)	14 (280)	29 (13)	0.05 (0.04)	44	0.05
	Responders (Day 2)	7 (60)	47 (19)	0.30 (0.08)	27	0.01
	Non-Responders (Day 2)	8 (155)	24 (13)	0.06 (0.08)	19	0.10

[1]5-12 day old, F_0 females sugar starved 24h
[2]SAI=Spatial Activity Index
[3]SR=Signed rank test statistic
[4]Probability that SAI value is equal to zero

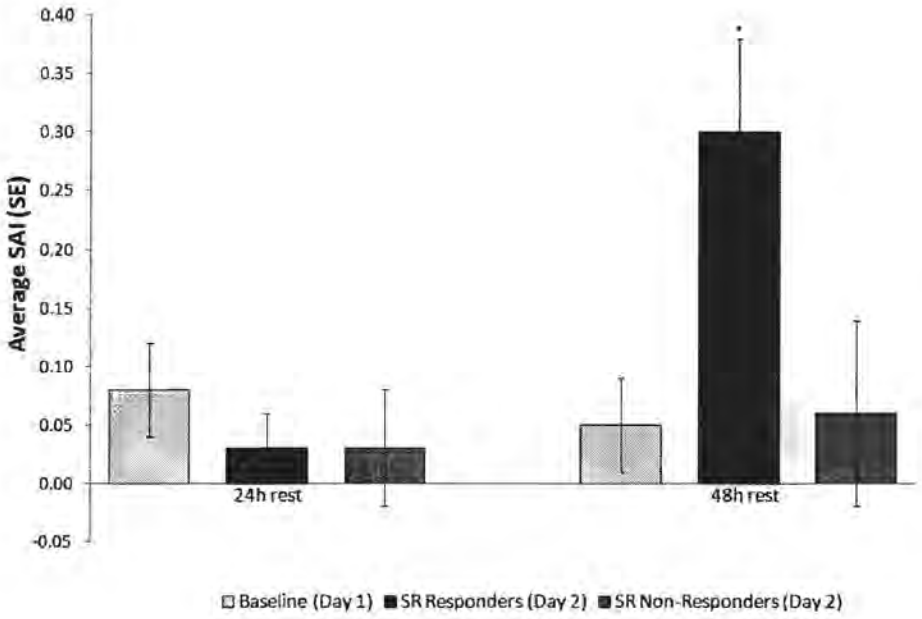


Figure 28. Plasticity of spatial repellency behaviors.
Weighted spatial activity index (SAI) scores for cohorts of *Aedes aegypti* females exposed to 1.35mg/m^3 transfluthrin. After observing baseline (Day 1) behaviors, test mosquitoes were re-assayed on a subsequent day following either 24 or 48 hours of resting. * indicates a day 2 SAI significantly different than the baseline day 1 SAI, $P<0.05$.

Table 11. Spatial repellency behaviors in selectively bred *Aedes aegypti*[1] responders (SRA+) and non-responders (SRA-).

Cohort	Number Trials (No. Mosqu.)	Mean Percent Active (SE)	Mean SAI[2] (SE)	SR[3]	p[4]	Cohort	Number Trials (No. Mosqu.)	Mean Percent Active (SE)	Mean SAI[2] (SE)	SR[3]	p[4]
F_0	9 (180)	29 (27)	0.14 (0.06)	21	0.02	F_0	9 (180)	29 (27)	0.14 (0.06)	21	0.02
SRA^+F_1	9 (180)	19 (14)	0.04 (0.03)	23	0.10	SRA^-F_1	9 (180)	26 (18)	0.03 (0.03)	16	0.11
SRA^+F_2	9 (180)	23 (11)	0.11 (0.04)	37	0.01	SRA^-F_2	9 (180)	9 (8)	0.02 (0.03)	6	0.34
SRA^+F_3	9 (180)	23 (14)	0.11 (0.03)	33	0.02	SRA^-F_3	9 (180)	10 (6)	0.02 (0.03)	11	0.29
SRA^+F_4	9 (180)	51 (13)	0.09 (0.04)	33	0.03	SRA^-F_4	9 (180)	36 (18)	-0.05 (0.04)	-20	0.13
SRA^+F_5	9 (180)	14 (12)	-0.02 (0.03)	-5	0.37	SRA^-F_5	9 (180)	22 (16)	-0.04 (0.05)	-8	0.32
SRA^+F_6	9 (180)	27 (11)	0.10 (0.03)	39	0.01	SRA^-F_6	9 (180)	25 (12)	-0.04 (0.03)	-21	0.10
SRA^+F_7	9 (180)	25 (11)	0.06 (0.05)	16	0.18	SRA^-F_7	9 (180)	34 (18)	0.02 (0.03)	7	0.29
SRA^+F_8	9 (180)	24 (17)	0.08 (0.02)	33	0.01	SRA^-F_8	9 (180)	28 (6)	-0.05 (0.02)	-33	0.10
SRA^+F_9	9 (180)	26 (10)	0.07 (0.02)	17	0.05	SRA^-F_9	9 (180)	31 (14)	-0.04 (0.05)	-13	0.25
						Experimental Cross[5]	9 (180)	30 (15)	0.11 (0.03)	27	0.02

[1]5-12 day old females, sugar starved 24h [2]SAI=Spatial Activity Index
[3]SR=Signed rank test statistic [4]Probability that SAI value is equal to zero
[5]Experimental cross between F9 SRA- females and F1 wt (unselected) males

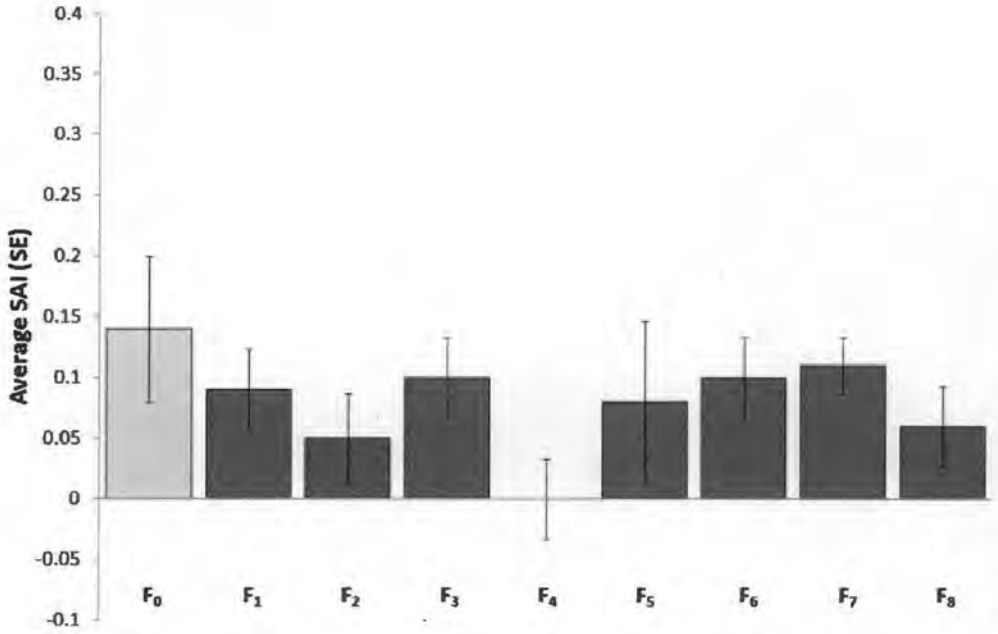


Figure 29. The maintenance of spatial repellent sensitivity in the freely mating *Ae. aegypti* colony.
Spatial activity index (SAI) values by generation in unselected (control) mosquitoes. There were no significant differences from the baseline in any generation (ANOVA with Dunnett's test for multiple comparisons, α=0.05).

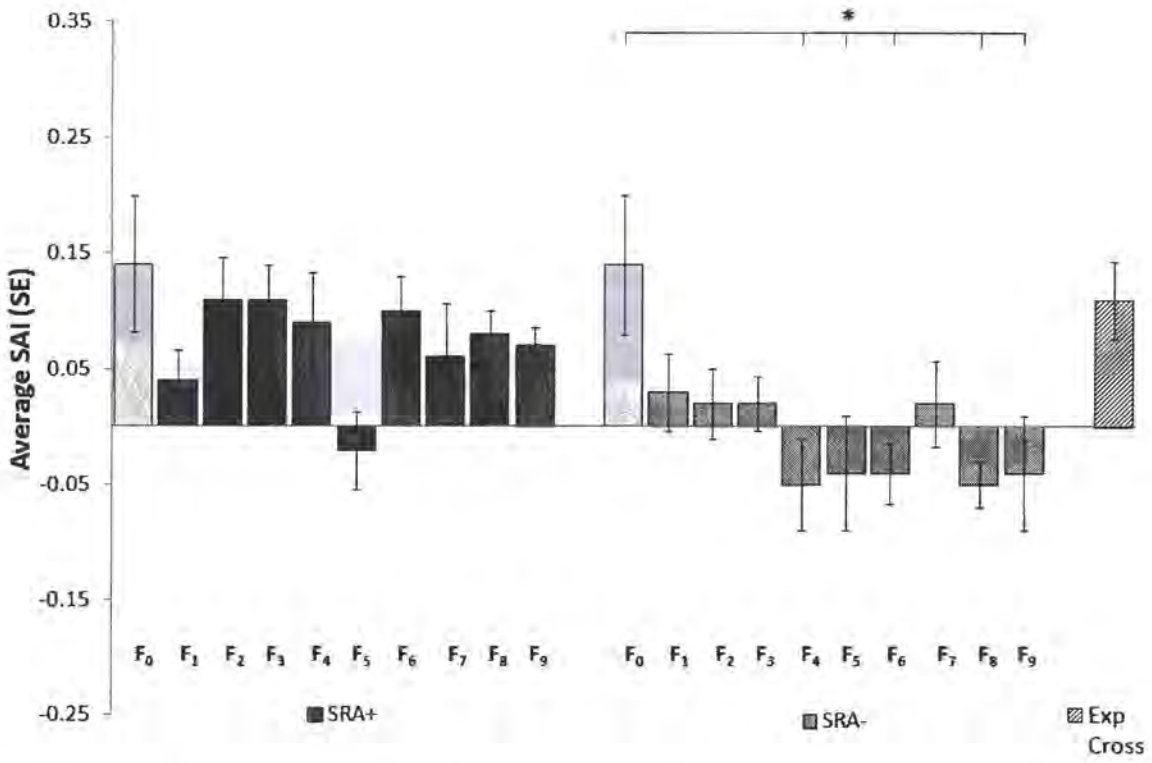


Figure 30. The heritability of spatial repellent insensitivity.
Spatial activity index (SAI) values by generation in selectively bred *Ae. aegypti* responder (SRA+) and non-responder (SRA-) strains. *= SAI values significantly different from the baseline F_1 generation via ANOVA with Dunnett's test for multiple comparisons, α=0.05. Exp Cross = F_9 SRA- females mates with F_1 wt males.

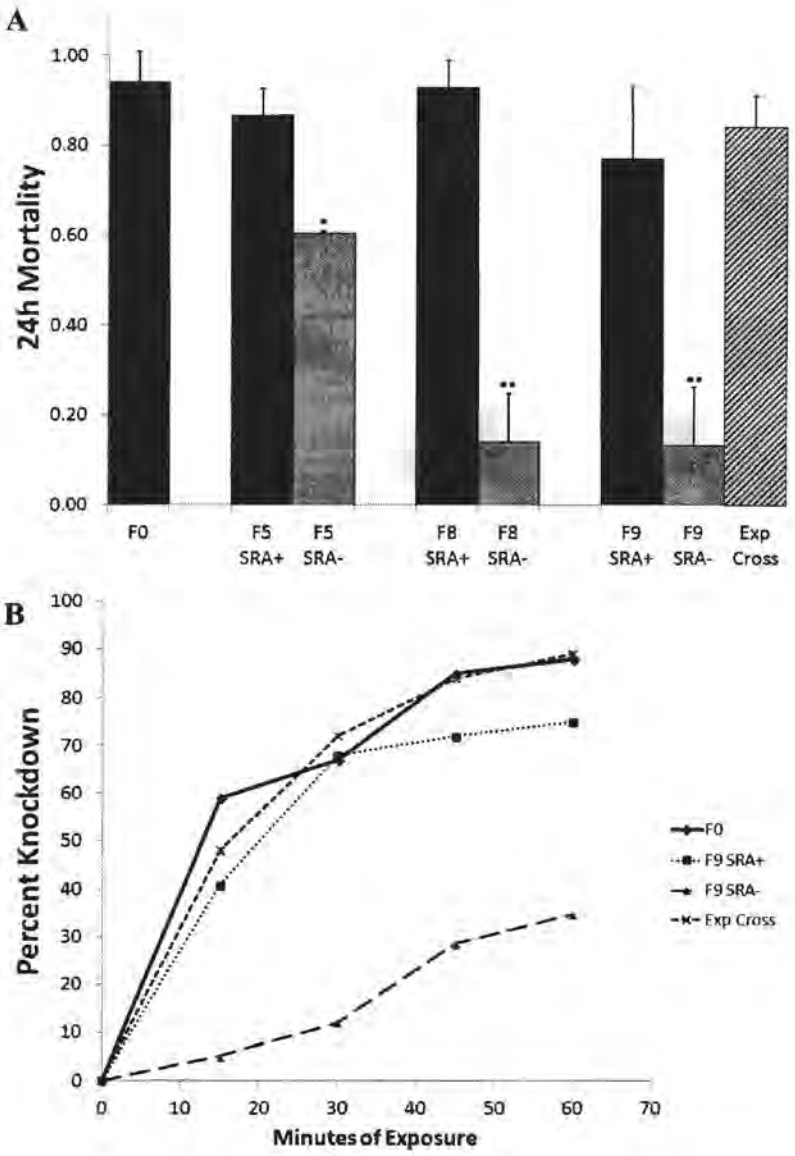


Figure 31. CDC bottle assay insecticide susceptibility patterns in selectively bred mosquito strains.
A) 24h mortality rates across various strains, asterisks signify significant differences from the baseline mortality rate * = $P<0.05$, **=$P<0.01$. B) Time to knockdown in a control (F1 unselected) strain and the F_9 generation of SR responders (SRA+) and non-responders (SRA-) and the experimental cross (F_8 SRA- females X F_1 wt males).

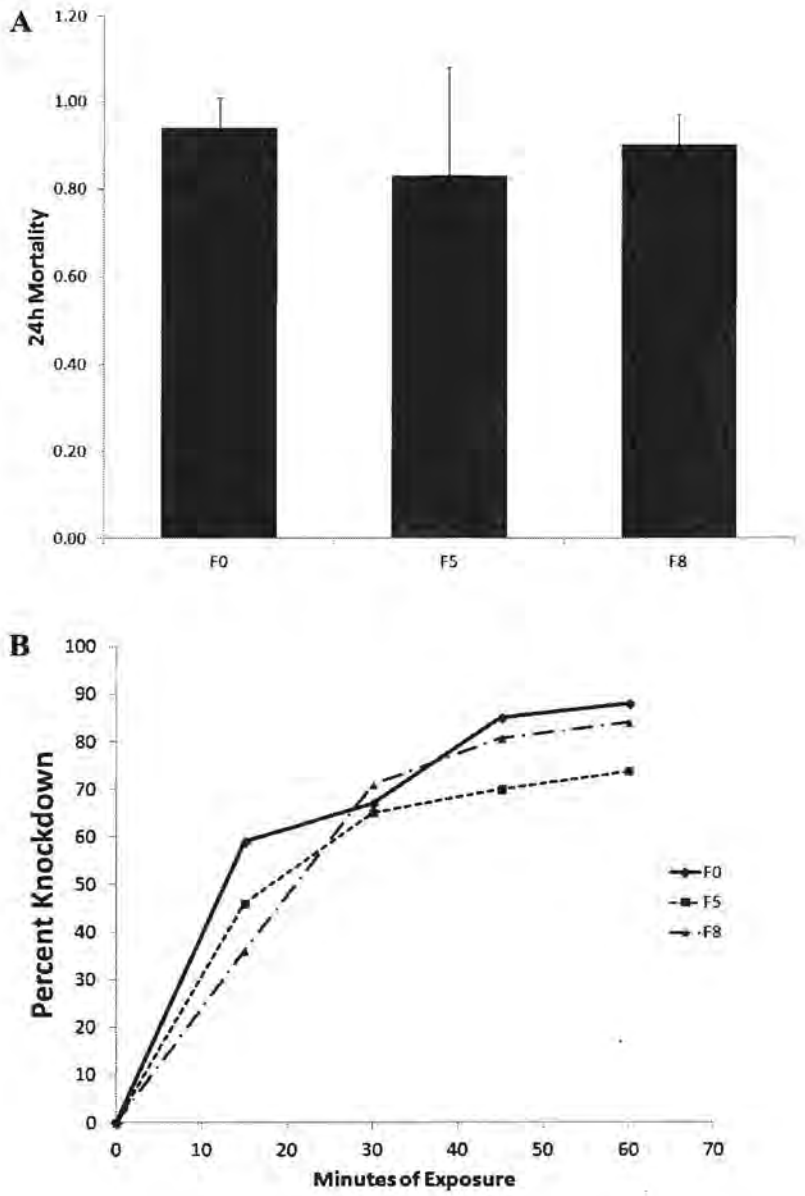


Figure 32. CDC bottle assay insecticide susceptibility patterns in the control mosquito population.
A) 24h mortality rates B) Time to knockdown.

CHAPTER 5: Summary and General Conclusions

"...the true objective of the measures applied is anti-malarial and not anti-mosquito...the yardstick for appreciation of results should be in terms of parasite incidence and not of vector densities." (54) – **Arnoldo Gabaldon,** 1978, on the feasibility of malaria elimination

DISSERTATION SUMMARY

Even as we move well into the 21st century, vector-borne diseases continue to impose an intolerable burden on human health (103; 159). While vector control interventions (actions that reduce contact between vectors and humans) can effectively, and sometimes dramatically, reduce the transmission of mosquito-borne pathogens in many ecological settings (149; 168), there is nonetheless a very real need to develop new tools and novel strategies to augment traditional approaches (59; 85; 167). Having identified combined push-pull strategies as one of the more promising new vector control paradigms under development (38; 111; 131; 147), the overall goal of the present work was to help further examine the potential for push-pull interventions to control mosquito vectors of human diseases. Key objectives included 1) evaluating the utility of the approach to control naturally occurring malaria vectors in northern Belize, Central America using an experimental hut methodology and 2) describing the plasticity, heritability and physiological basis of spatial repellency responses to transfluthrin in the global arbovirus vector *Aedes aegypti* using an *in vitro* behavioral bioassay.

THE EXPERIMENTAL UTILITY OF A PROTOTYPE PUSH-PULL INTERVENTION TO CONTROL NATURALLY OCCURRING MALARIA VECTORS IN NORTHERN BELIZE, CENTRAL AMERICA

The use of experimental huts to study natural mosquito behaviors in Belize was pioneered in the late 1990s by Bangs (19), Grieco (60), and Achee (5; 9), and their

continued use (130) has provided valuable insight into the biology of malaria transmission and control in this part of Central America. As detailed in chapters 2 and 3, we were fortunate to be able to build upon this foundation by utilizing multiple experimental huts at a field site near Progresso in Corozal District to concurrently assess the impact of the same experimental interventions on two different locally important malaria vectors, the more endophagic and anthropophagic *Anopheles vestitipennis* and the more exophagic and zoophagic *An. albimanus*.

Results from these studies are the first to show a combined effect on natural field populations of malaria vectors when using indoor spatial repellent strips and outdoor baited traps: for *An. vestitipennis*, the combined push-pull treatment simultaneously reduced mosquito entry into the huts and increased the efficacy of the outdoor traps. These results provide an exciting proof-of-concept, indicating that an optimized push-pull intervention targeting mosquitoes could reduce the probability of human-vector interactions both inside (by reducing mosquito entry) and outside (by increasing the efficacy of an outdoor baited trap) of homes. One critical observation, however, is the degree to which the underlying ecology of the target vector can impact intervention efficacy. For *An. albimanus*, a vector species that much more readily feeds outside and, given a choice, on non-humans, the same push-pull intervention produced a similar reduction in mosquito entry into the hut but no comparable increase in outdoor trap efficacies.

Bearing in mind the importance of a scientific proof-of-principle, it should be emphasized that these results show much work remains if the push-pull approach for mosquito control is to ever mature into viable public health interventions. The fact that

hanging bated CDC light traps outside the windows of a hut repeatedly (if insignificantly) reduced the spatial repellent effect of indoor transfluthrin emanators deserves further study. Questions worth investigating include whether or not this effect remains if the intervention utilizes a different repellent delivery mechanism, such as a commercially available product with optimally formulated active ingredient, and/or different trap types or positions. Additionally, the validation of the strategy in any locality will have to include rational selection of the best available tools in relation to that particular environment, and a thorough understanding of the local vector (and human) ecologies will be essential in shaping each intervention, e.g. which repellent products and which traps to use, and where to position them relative to the population at risk and to mosquito resting and feeding sites. Considering this, it is doubtful that an optimized push-pull intervention targeting *An. vestitipennis* will be exactly the same as an intervention tailored to target *An. albimanus*. Also, it is likely that in order to truly achieve maximum impact from a push-pull strategy we need to develop better traps and new repellents.

THE PLASTICITY, HERITABILITY AND PHYSIOLOGICAL MECHANISM OF SPATIAL REPELLENCY RESPONSES TO AIRBORNE TRANSFLUTHRIN IN *AEDES AEGYPTI*

The repellent used throughout this dissertation research was transfluthrin, a widely used volatile pyrethroid that, in addition to its proven spatial repellent efficacy against mosquitoes (48; 107; 110), is also highly toxic and irritating to susceptible mosquitoes (87; 105). Though the phenomenon of spatial repellency has been widely documented and observed (63; 122), the fundamental physiological processes that produce SR behaviors are poorly understood (8; 22). Elucidating which mechanisms produce SR behaviors in specific transmission settings is an important step to understanding how to best use spatial repellents in a public health context (106), how

their long-term use might impact vector populations over time (63; 87) and could also aid in the rational design of new active ingredients (8). As described in chapter 4, we expanded the use of an established *in vitro* behavioral bioassay developed in our laboratory (65) to not only observe and quantify SR behaviors, but also, for the first time, to track changes in SR behavioral responses to transfluthrin over time and throughout selectively bred generations.

Results from these *in vitro* studies have produced several novel insights, primarily into the fundamental nature of SR behaviors in *Aedes aegypti* exposed to airborne transfluthrin. First, and not surprisingly, the behaviors that we observed in our bioassay system were relatively plastic, indicating that spatial repellency is a complex behavior that likely results from a combination of factors, some heritable and others non-heritable. Secondly, and perhaps more interestingly, we also provided evidence that one of the primary mechanisms by which transfluthrin can elicit SR behaviors in *Ae. aegypti* is through physiological irritation of the mosquito by sub-lethal doses of insecticide vapors, apparently acting via the same neurotoxic pathways (i.e. by binding to voltage-gated sodium ion channels and disrupting neuronal action potential propagation) that can result in knockdown and death at higher doses. One point worth emphasizing is that our experimental design did not investigate the possibility that volatile transfluthrin might also act through olfactory or gustatory means, either by active processes or by masking the presence of a blood meal, and these questions also warrant further investigation.

Our *in vitro* work is also notable for having experimentally reduced insecticide susceptibility in a selectively bred population of vectors that were exposed only to sub-lethal doses of an airborne pyrethroid. This raises some important questions about how

long term or repeated exposures to sub-lethal amounts of a toxic chemical might impact the repellent sensitivity and/or insecticide susceptibility of target vectors. One of the potential befits of expanding the public health use of repellents is the opportunity to reduce our reliance on traditional vector control approaches that prioritize the direct killing of vectors, thereby reducing the selective pressures that have resulted in widespread insecticide resistance (6; 8; 29). Though care should be taken not to interpret our preliminary results as necessarily epidemiologically or operationally relevant, we should nonetheless be aware of the possibility that exploiting the behavior modifying effects of chemicals that are also, at higher doses, toxic may not completely eliminate these selective pressures. This observation is especially concerning when viewed against the current backdrop of worldwide reliance on pyrethroids as the primary insecticide class for public health and agricultural applications and the accompanying concerns about the rapid expansion of pyrethroid resistance in key vector species (6; 8; 70; 116). The effect of introducing of a volatile pyrethroid spatial repellent in a location where residual pyrethroids are already in use is uncertain and warrants careful consideration and further evaluation and monitoring. This uncertainty also highlights both the need to better understand the physiological mechanisms that drive SR behaviors and the pressing requirement to identify and develop new active ingredients with novel mechanisms action unrelated to current intervention standards.

CONCLUDING REMARKS

Whether one considers a disease like malaria, which has afflicted humanity throughout our entire evolutionary history, or more recently emergent threats like the viruses that cause dengue fever and chikungunya, the truth is that the ecology of vector-

borne diseases is extraordinarily complex and dynamic and, even when effective tools are available, they often prove difficult to control. Additionally, in many parts of the world current vector control successes are being threatened as the best available tools are either failing (e.g., insecticide resistance, shifting vector statuses) and/or increasingly difficult to implement (e.g., ecologically, politically). This introduces even more complexity to the situation and adds a sense of urgency to the need to develop new vector control paradigms.

My hope is that the work presented here, while preliminary in nature, contributes to a larger body of evidence that supports further investment into the optimization and validation of the use of spatial repellents in public health, including 1) the continued development of sustainable push-pull strategies for malaria vector control and 2) the identification of new insect behavior modifying compounds with novel mechanisms of action unrelated to any of the chemical classes currently used in vector control. Collectively, our results paint an encouraging picture, but the story is incomplete: proof-of-principle studies must validate the push-pull approach in varied and diverse transmission settings and we need new active ingredients, along with a better understanding of how repellents work and how their long term use might affect target vector populations over time.

APPENDIX A: Protocol Approvals

MINISTRY OF HEALTH, BELIZE: INSTITUTIONAL REVIEW BOARD

Ministry of Health
Institutional Review Board

9th January, 2012

Joseph M. Wagman
PhD Candidate
Emerging Infectious Diseases Program
Uniformed Services University of the Health Sciences
Bethesda, MD 20814 USA
Email: Joseph.Wagman@usuhs.mil

Re: Research Protocol "Spatial Repellency and the Experimental Utility of a Push-Pull Mosquito Control Strategy for the Prevention of Malaria"

IRB Tracking: 01/12(02)

Dear Mr. Wagman:

The Institutional Review Board (IRB) of the Ministry of Health, Belize hereby approves the research protocol entitled ***Spatial Repellency and the Experimental Utility of a Push-Pull Mosquito Control Strategy for the Prevention of Malaria*** to be executed as described in the submitted protocol and letter of amendment dated January 7th, 2012 and according the conditions set out in the attached addendum.

Regards,

MICHAEL. PITTS
Signature/Name, Director of Health Services
Date 9/1/12

AISHA ANDREWS
Signature/Name, Board Member
Date 9/01/12

DR. JORGE BOLANOS
Signature/Name, Board Member
Date 9-JAN-12

Joseph M Wagman
Signature/Name, Lead Investigator
Date 11 JAN 2012

ADDENDUM

CONDITIONS OF RESEARCH

Subsequent to the approval of the Institutional Review Board, Ministry of Health, Belize, the lead investigator(s) is/are responsible for the following:

a) conduct the research project as set forth in the protocol;

b) draw up and submit partial and final reports;

c) submit any data requested by the IRB, at any time;

d) keep in a file, under his/her guard, for five years, all research data, including individual records and all other documents recommended by the IRB;

e) submit the results for publication, with due credit given to the associate researchers and technical personnel participating in the project; and

f) justify to the IRB the interruption of the project or the non-publication of its results.

PESTICIDES CONTROL BOARD, BELIZE

Pesticides Control Board, Belize

1988 - 2008

20 years promoting rational pesticide management!

August 31, 2012

Ref: PCB/EXP/MOH/01/12

Comptroller of Customs
Department of Customs & Excise
P.O. Box 146
Belize City

Dear Sir,

Authorization to import pesticides not registered in the Register of Pesticides

This is to authorize **Nicole Achee b/o Joseph Wagman for the Ministry of Health** to import the following pesticide, in the following quantity, under Regulation VI (1 & 2) and Regulation VII of the Registered and Restricted Pesticides (Registration) Regulations, 1995.

Bayothrin *(transfluthrin)* ..... 1kg.

The purpose and location (Progresso, Corozal District) for the use of this product has been communicated to the Board as required in these Regulations.

Sincerely,

for Miriam Serrut, MA
Registrar of Pesticides

PESTICIDES CONTROL BOARD

c.c. Edgar Silva/Raul Vera
BAHA-Quarantine Dept, PBL

Central Farm, Cayo District, Belize, Central America
Tel: 501-824-2640 Fax: 501-824-3486 E-mail: pcbinfo@btl.net
www.pcbbelize.com

PESTICIDES CONTROL BOARD

Permit for Importation and Use of Experimental Pesticides

This is to certify that permission has been conditionally granted to **Nicole Achee b/o Joseph Wagman for the Ministry of Health** to import the herbicide **Bayothrin *(transfluthrin)*** on an experimental basis. The experimental trial is to be performed by **Uniformed Services University of the Health Sciences** for application in **Huts located south of Progresso Village,** in the **Corozal District.**

Such permission has been given subject to the following conditions:

1. Importer shall notify the Pesticides Control Board of the commencement date of the experimental trial. Results of the trial shall also be forwarded to the **PCB.**
2. The experimental pesticide will not be offered for sale in Belize by the institution without prior approval from the **PCB.**
3. The institution shall conduct the trial in an environmentally sound matter.
4. The institution shall be held responsible for any unauthorized use of this pesticide on other crops or area.
5. The institution shall not offer for sale any product grown in the experimental plots during the trial without prior approval from the Pesticide Control Board.
6. The Pesticide Control Board will have the right to discontinue any ongoing trial if the institution is found to have contravened any of the above conditions.
7. The applicant shall cover food, transport and accommodation expenses for monitoring of the trial by Board personnel (where necessary).

Signed this 31 day of August, 2012 at Central Farm, Cayo.

PESTICIDES CONTROL BOARD

Miriam Serrut, MA
Registrar
Pesticides Control Board

Central Farm, Cayo District, Belize, Central America
Tel: 501-824-2640 Fax: 501-824-3486 E-mail: pcbinfo@btl.net
www.pcbbelize.com

APPENDIX B: Data Collection Sheets

Progreson Hut Site: Mosquito Entry Data Sheet

Date:	Hut:	Experiment:	Treatment:	Collection Team:			

Collection	Time	WA	WB	WC	In temp	In RH	Initials	Notes
1	5:30 PM							
2	6:00 PM							
3	6:30 PM							
4	7:00 PM							
5	7:30 PM							
6	8:00 PM							
7	8:30 PM							
8	9:00 PM							
9	9:30 PM							
10	10:00 PM							
11	10:30 PM							
12	11:00 PM							
13	11:30 PM							

Progresso Hat Site: Mosquito Entry Data Sheet

Date:	Hut:	Experiment:	Treatment:	Collection Team:			

Collection	Time	WA	W3	WC	In temp	In RH	Initials	Notes
14	12:00 AM							
15	12:30 AM							
16	1:00 AM							
17	1:30 AM							
18	2:00 AM							
19	2:30 AM							
20	3:00 AM							
21	3:30 AM							
22	4:00 AM							
23	4:30 AM							
24	5:00 AM							
25	5:30 AM							
26	6:00 AM							

PARITY SLIDES

Slide #	Date						
	Species						
	Location						
	Window						

Slide #	Date						
	Species						
	Location						
	Window						

Slide #	Date						
	Species						
	Location						
	Window						

Slide #	Date						
	Species						
	Location						
	Window						

Slide #	Date						
	Species						
	Location						
	Window						

Slide #	Date						
	Species						
	Location						
	Window						

Slide #	Date						
	Species						
	Location						
	Window						

Progresso Hut Collection Pre-departure Checklist

Collection Coolers (4)	Coolers (4)	x
	Collection Cups and lids	x
	Killing Chamber	x
	Acetone & Cotton	x
	Forceps	x
	Petry Dishes (2)	x
	Sharpie Marker	x
	MaGNIFYING Glass	x
	Collection Tubes	x
Big Cooler	Aspirators	x
	Extra Cotton	x
	Acetone	x
	Data Sheets & Pen	x
	Disecting Kit	x
	Plastic Bags, labeled, for tubes	x
	Microscope	x
	Microscope light source	x
	Nicole's Field Box	x
	Outdoor Traps & Batteries	x
	Outdoor Trap Replacement Bags	x
	Duct Tape	x
	Masking Tape	x
	Radio	x
	Tubies	x
	Sharpie Marker	x
	Petry Dishes (2)	x
	Alcohol	x
	Paper Towels	x
	SR Nets: Control	x
	SR Nets: Trans	x
	Candles	x
	Slide Drying Box	x

Backpack	Flash Lights & Batteries	x
	Tape Measure	x
	Hobos	x
	Hobo Shuttle	x
	Hand Counter	x
	Toolbox	x
Truck	Window Traps	x
	Lighters	x
	Anameter	x
	Water	x
	Blankets	x
	Mosquito Nets	x
	Tarps	x
	Drill	x

USUHS/MoH Belize Larval Survey Identification Sheet	
Date:	
Location:	
Container Type:	
GPS Coordinates:	
Total Larvae Collected:	
Larvae Identified:	
Adults Identified:	

USUHS/MoH Belize Larval Survey Identification Sheet	
Date:	
Location:	
Container Type:	
GPS Coordinates:	
Total Larvae Collected:	
Larvae Identified:	
Adults Identified:	

Test Date: Tested By:

Treatment	Chambers	REP	N	CCNT	TCNT	KD CLEAR	KD CON	KD TRT	TEMP	HUM	TIME	GEN/AGE	KD PRIOR	COMMENTS
	C1:T1	1												
	C1:T1	2												
	C1:T1	3												
	C1:T1	4												
	C1:T1	5												
	C1:T1	6												
	C1:T1	7												
	C1:T1	8												
	C1:T1	9												
	C2:T2	1												
	C2:T2	2												
	C2:T2	3												
	C2:T2	4												
	C2:T2	5												
	C2:T2	6												
	C2:T2	7												
	C2:T2	8												
	C2:T2	9												
	C3:T3	1												
	C3:T3	2												
	C3:T3	3												
	C3:T3	4												
	C3:T3	5												
	C3:T3	6												
	C3:T3	7												
	C3:T3	8												
	C3:T3	9												
	C4:T4	1												
	C4:T4	2												
	C4:T4	3												
	C4:T4	4												
	C4:T4	5												
	C4:T4	6												
	C4:T4	7												
	C4:T4	8												
	C4:T4	9												

CDC Bottle Assay Data Sheets_1.xlsx

Date Species/strain Notes:

Chemical TEMP
Dose RH

	Bottle 1		Bottle 2		Bottle 3		Bottle 4		Total		Control	
Time (min)	Alive	Dead	Alive	Dead	Alive	Dead	Alive	Dead	Alive	Dead	Alive	Dead
0												
15												
30												
45												
60												
24 hr												

Date Species/strain Notes:

Chemical TEMP
Dose RH

	Bottle 1		Bottle 2		Bottle 3		Bottle 4		Total		Control	
Time (min)	Alive	Dead	Alive	Dead	Alive	Dead	Alive	Dead	Alive	Dead	Alive	Dead
0												
15												
30												
45												
60												
24 hr												

APPENDIX C: Latin square Rotation Schedules, Progresso Hut Site, Belize

		Hut 2	Hut 3
Night 1	Intervention	CDC	BG
	Collection Teams	1	2
Night 2	Intervention	CDC	BG
	Collection Teams	2	1
Night 3	Intervention	BG	CDC
	Collection Teams	1	2
Night 4	Intervention	BG	CDC
	Collection Teams	2	1

		Hut 1	Hut 2	Hut 3	Hut 4
Night 1	Intervention	Control	Pull	SR	PP
	Collection Team	1	2	3	4
Night 2	Intervention	Control	Pull	SR	PP
	Collection Team	4	1	2	3
Night 3	Intervention	Control	Pull	SR	PP
	Collection Team	3	4	1	2
Night 4	Intervention	Control	Pull	SR	PP
	Collection Team	2	3	4	1
Night 5	Intervention	PP	Control	Pull	SR
	Collection Team	4	3	2	1
Night 6	Intervention	PP	Control	Pull	SR
	Collection Team	1	4	3	2
Night 7	Intervention	PP	Control	Pull	SR
	Collection Team	2	1	4	3
Night 8	Intervention	PP	Control	Pull	SR
	Collection Team	3	2	1	4
Night 9	Intervention	SR	PP	Control	Pull
	Collection Team	1	2	3	4
Night 10	Intervention	SR	PP	Control	Pull
	Collection Team	4	1	2	3
Night 11	Intervention	SR	PP	Control	Pull
	Collection Team	3	4	1	2
Night 12	Intervention	SR	PP	Control	Pull
	Collection Team	2	3	4	1
Night 13	Intervention	Pull	SR	PP	Control
	Collection Team	4	3	2	1
Night 14	Intervention	Pull	SR	PP	Control
	Collection Team	1	4	3	2
Night 15	Intervention	Pull	SR	PP	Control
	Collection Team	2	1	4	3
Night 16	Intervention	Pull	SR	PP	Control
	Collection Team	3	2	1	4

APPENDIX D: Auxiliary Observations on Transfluthrin Susceptibility in *Anopheles vestitipennis* from Progresso Hut Site, Belize

In order to roughly characterize the transfluthrin susceptibility of Anopheline mosquitoes at the Progresso Hut Site in Northern Belize, some limited CDC bottle bioassays were performed at three time points, before (28 August), during (13 October) and near to the end (27 November) of the 2012 push-pull experimental hut evaluations. In short, a mixed field population of adult female mosquitoes was collected via human landing collection at or near one of the experimental huts, stored in collection buckets with access to cotton gauze soaked in a 10% sucrose solution, and transported to the Orange Walk field laboratory in a humidified chamber. On the following day, transfluthrin susceptibility was assayed following the standard CDC bottle test protocol (24) using a diagnostic dose of 6.25 μg transfluthrin per bottle. Only *An. vestitipennis* were collected in sufficient numbers for testing (August n=40; October n=39, November n=33). Results indicated a sustained high susceptibility to transfluthrin toxicity at each experimental time point, with greater than 95% corrected mortality by 45 minutes observed on each date (Figure A1).

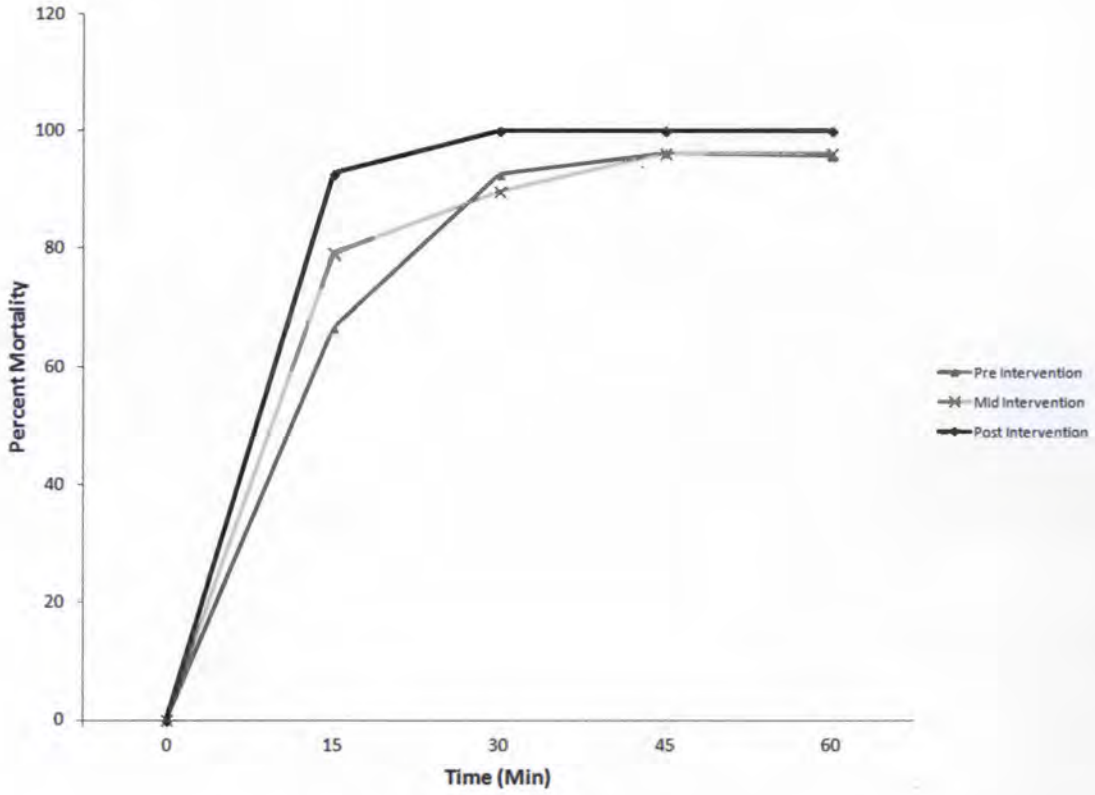


Figure A1. Field caught *An. vestitipennis* susceptibility to transfluthrin killing. Measured using field caught mosquitoes from human landing collections at the Progresso Hut Site and a standard CDC bottle test approach with 6.25 µg transfluthrin per bottle.

APPENDIX E: Auxiliary Observations on *Anopheles* spp. Knockdown and Mortality Inside Repellent-Treated Experimental Huts

During the experimental hut studies that employed passively emanating transfluthrin as indoor spatial repellent, casual observations of the numbers of anopheline mosquitoes that were knocked down (KD), both inside window intercept traps or otherwise freely flying indoors, were made at each hut (Table A1). Numbers were aggregated into huts with repellent (SR) and huts with no repellent (Control). Observations were strictly secondary to the primary work of collecting and processing mosquitoes from the intercept traps and should not be over-interpreted, but do indicate that 1) more KD was observed inside huts with transfluthrin emanators but 2) overall, the KD rate inside SR huts was still lower than expected (less than 1% of all mosquitoes collected) given how highly toxic transfluthrin is to mosquitoes (48). While this data seems to provide confidence that the manufactures recommended dosing and delivery scheme (89) is appropriate for evaluating spatial repellency, the true knockdown and mortality effects of low doses of transfluthrin that are intended to be sub-lethal is an area worth further investigation, especially in field-based 'operational' settings.

Anecdotally, we also noticed a temporal trend in which increased indoor KD seemed to correlate with colder temperatures. Whether or not this trend would hold true if properly tested is an interesting question worth studying, and could provide insight into how meteorological factors can impact vector control intervention success by altering both the physical dispersion of active ingredient and the physiological status of the target organisms.

Table A1. Indoor *Anopheles* spp. knockdown noted during the push-pull experimental hut evaluations.

	KD[1] in window intercept traps		KD inside huts		Total Mosqutioes Collected		Rough KD rate estimates	
Date	SR[2]	Control[3]	SR	Control	SR	Control	SR	Control
3-Sep	0	0	3	0	161	453	0.019	0.000
6-Sep	0	0	1	0	50	241	0.020	0.000
10-Sep	0	0	1	0	49	476	0.020	0.000
13-Sep	2	0	2	0	30	183	0.067	0.000
17-Sep	0	0	0	0	51	130	0.000	0.000
24-Sep	2	0	3	0	173	417	0.017	0.000
27-Sep	0	0	3	0	40	59	0.075	0.000
4-Oct	0	0	1	1	207	346	0.005	0.003
8-Oct	0	0	0	0	95	168	0.000	0.000
11-Oct	2	0	3	0	199	375	0.015	0.000
14-Oct	0	0	4	1	551	677	0.007	0.001
18-Oct	1	1	5	2	206	310	0.024	0.006
22-Oct	0	0	6	0	248	316	0.024	0.000
25-Oct	0	0	0	0	23	240	0.000	0.000
22-Nov	0	0	0	0	19	72	0.000	0.000
26-Nov	0	0	0	0	94	73	0.000	0.000
Total	**7**	**1**	**32**	**4**	**2196**	**4536**	**0.0146**	**0.0009**

[1]KD= Mosquito Knockdown
[2]SR = Experimental huts with spatial repellent
[3]Control = Experimental huts with no repellent

APPENDIX F: First Record and Demonstration of a Southward Expansion of *Aedes albopictus* into Orange Walk Town, Belize, Central America

Journal of the American Mosquito Control Association, 29(4):380–382, 2013

SCIENTIFIC NOTE

FIRST RECORD AND DEMONSTRATION OF A SOUTHWARD EXPANSION OF *AEDES ALBOPICTUS* INTO ORANGE WALK TOWN, BELIZE, CENTRAL AMERICA[1]

JOSEPH WAGMAN,[2] JOHN P. GRIECO,[2] RUSSELL KING,[3] IRENEO BRICEÑO,[3] KIM BAUTISTA,[3] JORGE POLANCO,[3] JAMES PECOR[4] AND NICOLE L. ACHEE[2]

ABSTRACT. The first record of *Aedes albopictus* in northern Belize was made in Orange Walk Town, Orange Walk District, on November 3, 2011. *Aedes* spp. larvae were collected during a routine Ministry of Health mosquito survey and reared to adults. Upon emergence, a mixed population of *Aedes aegypti* (35) and *Ae. albopictus* (11) was observed (*aegypti*:*albopictus* = 3:1). Subsequent larval and adult surveys in Orange Walk and Corozal District, also in northern Belize, have confirmed the presence of *Ae. albopictus*, thereby indicating the range expansion and establishment of this nuisance biter and potential disease vector in Belize.

KEY WORDS *Aedes albopictus*, Asian tiger mosquito, distribution, Belize, Central America

The global expansion of *Aedes albopictus* (Skuse) from its native habitat in Asia to far-reaching areas of the world has been well documented (Benedict et al. 2007, Enserink 2008, Lambrechts et al. 2010). There are many reasons to monitor the expanding geographical footprint of this notoriously invasive species, among which is its reputation as a nuisance biter and its potential to vector several viral pathogens of public health importance, including dengue and chikungunya viruses (Moore and Mitchell 1997, Lambrechts et al. 2010).

The current spread of *Ae. albopictus* into the western hemisphere was first reported from specimens collected in Houston, TX, in 1985 after a shipment of used tires harboring mosquito eggs arrived from Japan (Craven et al. 1988). Since then, the mosquito has either expanded in range outward from Texas or been similarly introduced throughout the Americas. By 1995, this range included every country that shares a terrestrial border with the country of Belize (Fig. 1; Mexico: 1988, Guatemala: 1995, and Honduras: 1995) (Ogata and Samayoa 1996, Benedict et al. 2007; Ortega-Morales et al. 2010, Salomon-Grajales et al. 2012). The first record of *Ae. albopictus* in Belize was noted during 2009, when an adult female mosquito was captured after having landed on public health personnel checking oviposition traps in Benque Viejo del Carmen, a town in the western district of Cayo that borders the department of Petén in Guatemala (Ortega-Morales et al. 2010). Since that time, however, no new records of *Ae albopictus* have been documented in Belize.

On November 3, 2011, *Aedes* spp. larvae collected by Ministry of Health (MoH) personnel during routine dengue virus prevention activities were brought to the Uniformed Services University of the Health Sciences (USU) field insectary in Orange Walk Town, Belize. Larvae were then reared to adults and identified to species using species-specific keys (Rueda 2004). At a private house in Orange Walk District (18°04′11.64″N, 88°33′32.39″W), 46 larvae were collected from a 19-liter plastic bucket used for outdoor water storage, of which 35 adults were later identified as *Aedes aegypti* (L.) and 11 as *Ae. albopictus*. Subsequent collections of larvae, during MoH surveillance, and adults, via human landing capture during USU laboratory activities, from November 2011 to February 2012 indicated the presence of *Ae. albopictus* in at least 5 other locations throughout Orange Walk and Corozal districts (Table 1). At each *Ae. albopictus*–positive location, a mixed population of both *Aedes* species, with *Ae. aegypti* predominating in an approximate ratio of 4:1, was observed. *Aedes albopictus* larvae from Orange Walk Town were reared to adults, and F_1 specimens were sent to the Walter Reed Biosystematics Unit at the Smithsonian Institution for species confirmation and specimen archiving.

While the impact on disease transmission, if any, of the arrival of *Ae. albopictus* in northern Belize is unclear, it is important to note that

[1] The opinions contained herein are the private views of the authors and are not to be construed as official or reflecting the views of the Department of Defense, the Uniformed Services University of the Health Sciences, or the Walter Reed Army Institute of Research.

[2] Department of Preventive Medicine and Biometrics, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Road, Bethesda, MD 20814.

[3] Ministry of Health, East Block Independence Plaza, Belmopan, Belize, Central America.

[4] Walter Reed Biosystematics Unit, 4210 Silver Hill Road, Suitland, MD 20746.

Table 1. *Aedes albopictus* collection sites in northern Belize, 2011–12.

Site	Collection date	Location	District	Position	Container type	Total collected		Ratio
						Ae. aegypti	*Ae. albopictus*	
A	Nov. 3, 2011	Orange Walk Town	Orange Walk	18°04.194′N, 88°33.540′W	19-liter plastic bucket	35	11	3.2:1
B	Nov. 7, 2011	Orange Walk Town	Orange Walk	18°04.316′N, 88°33.710′W	3-liter cooking pot	19	4	4.8:1
C	Nov. 9, 2011	Orange Walk Town	Orange Walk	18°04.938′N, 88°33.390′W	Tire	40	9	4.4:1
D	Dec. 14, 2011	Orange Walk Town	Orange Walk	18°04.938′N, 88°33.390′W	Adult landing capture	9	4	2.3:1
E	Feb. 9, 2012	Louisville	Corozal	18°19.180′N, 88°30.305′W	Tire	42	7	6.0:1
F	Feb. 9, 2012	Cristo Rey	Corozal	18°20.990′N, 88°29.695′W	200-liter plastic drum	33	9	3.7:1
G	Feb. 22, 2012	San Pablo	Orange Walk	18°12.721′N, 88°33.756′W	Tire	50	14	3.6:1

dengue virus is endemic throughout Belize (>1,500 clinical cases per annum reported for 2009–12 [Bautista 2012]), and case monitoring and control is considered a high priority for both the public health and tourism sectors (Vanzie 2008, Bautista 2012). In Belize, increased and continued surveillance of the expanding range of *Ae. albopictus* could provide an opportunity to measure whether or not *Ae. albopictus* might displace natural populations of *Ae. aegypti* in the region, as has been previously observed in some parts of the Americas but not others (Hornby et al. 1994, Estrada-Franco and Craig 1995, Juliano 2010, Eisen and Moore 2013). Additionally, further evaluation of the vector status and insecticide susceptibility for regional populations of *Ae. albopictus* can help elucidate its role in natural disease transmission in Central America and better inform disease prevention activities.

REFERENCES CITED

Bautista K. 2012. Vector control workshop: opening remarks. In: Wagman J, ed. *Vector control workshop*. San Ignacio Town, Cayo District, Belize: Ministry of Health. p 16–20.

Benedict MQ, Levine RS, Hawley WA, Lounibos LP. 2007. Spread of the tiger: global risk of invasion by the mosquito *Aedes albopictus*. *Vector Borne Zoonotic Dis* 7:76–85.

Craven RB, Eliason DA, Francy DB, Reiter P, Campos EG, Jakob WL, Smith GC, Bozzi CJ, Moore CG, Maupin GO, Monath TP. 1988. Importation of *Aedes albopictus* and other exotic mosquito species into the United States in used tires from Asia. *J Am Mosq Control Assoc* 4:138–142.

Eisen L, Moore CG. 2013. *Aedes* (*Stegomyia*) *aegypti* in the continental United States: a vector at the cool margin of its geographic range. *J Med Entomol* 50:467–478.

Enserink M. 2008. A mosquito goes global. *Science* 320:864–866.

Estrada-Franco JG, Craig GB. 1995. Biology, disease relationships, and control of *Aedes albopictus*. In: PAHO. *Technical paper No. 24*. Washington, DC: Pan American Health Organization. p 24–27.

Hornby JA, Moore DE, Miller TW Jr. *Aedes albopictus* distribution, abundance, and colonization in Lee County, Florida, and its effect on *Aedes aegypti*. *J Am Mosq Control Assoc* 10:397–402.

Juliano SA. 2010. Coexistence, exclusion, or neutrality? A meta-analysis of competition between and resident mosquitoes. *Isr J Ecol Evol* 56:325–351.

Lambrechts L, Scott TW, Gubler DJ. 2010. Consequences of the expanding global distribution of *Aedes albopictus* for dengue virus transmission. *PLoS Negl Trop Dis* 4:e646.

Moore CG, Mitchell CJ. 1997. *Aedes albopictus* in the United States: ten-year presence and public health implications. *Emerg Infect Dis* 3:329–334.

Ogata K, Samayoa AL. 1995. Discovery of *Aedes albopictus* in Guatemala. *J Am Mosq Control Assoc* 12:503–506.

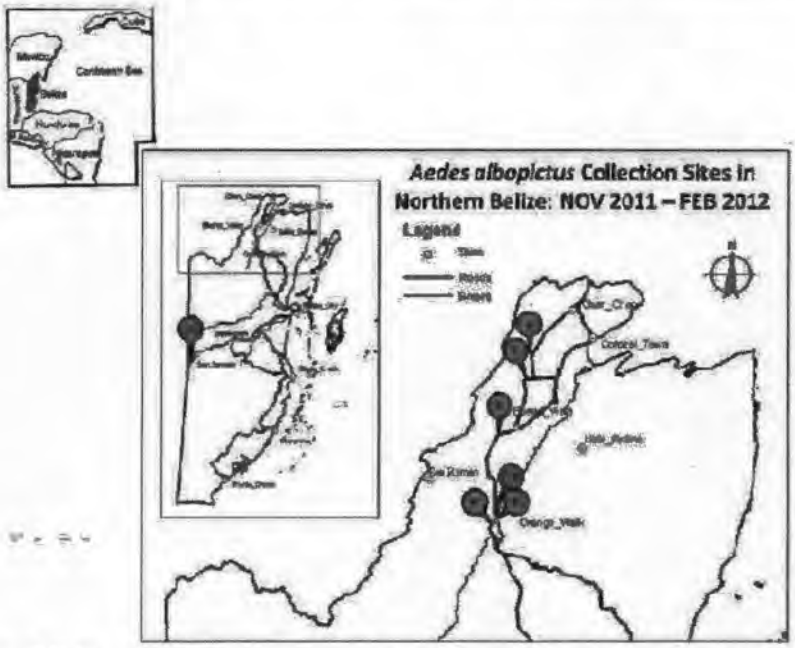


Fig. 1. Map of Belize, with the locations described in Table 1 indicated. The initial description of *Aedes albopictus* in Belize (Ortega-Morales et al. 2010) occurred near San Ignacio along the western border, indicated in the map inset.

Ortega-Morales AI, Mis-Avila P, Dominguez-Galera M, Canul-Amaro G, Esparza-Aguilar J, Carlos-Azueta J, Badillo-Perry S, Marin P, Polanco J, Fernandez-Salas I. 2010. First record of *Stegomyia albopicta* [*Aedes albopictus*] in Belize. *Southwest Entomol* 35:197–198.

Rueda LM. 2004. *Pictorial keys for the identification of mosquitoes (Diptera: Culicidae) associated with dengue virus transmission.* Auckland, New Zealand: Magnolia Press.

Salomon-Grajales J, Lugo-Moguel GV, Tinal-Gordillo VR, de la Cruz-Velazquez J, Beaty BJ, Eisen L, Lozano-Fuentes S, Moore CG, Garcia-Rejon J. 2012. *Aedes albopictus* mosquitoes, Yucatan Peninsula, Mexico. *Emerg Infect Dis* 18:525–527.

Vanzie E. 2008. Assessment of the impact of climate change on Belize's health sector: dengue and dengue hemorrhagic fever. Belmopan, Belize: Ministry of Health, Belize.

REFERENCES

1. 1993. *Guyana and Belize: country studies*. Washington, DC: Federal Research Division, US Library of Congress. 408 pp.

2. 2008. Environmental Health Program Strategic Plan 2009-2011, Ministry of Health, Belmopan, Belize

3. 2013. 2010 Population and Housing Census Report, Statistical Institute of Belize, Belmopan, Beize

4. 2014. *Central America and Caribbean: Belize.* https://www.cia.gov/library/publications/the-world-factbook/geos/bh.html

5. Achee N. 2004. *A study on the bionomics of* Anopheles darlingi *Root (Diptera:Culicidae) in Belize, Central America*. Uniformed Services University of the Health Sciences, Bethesda, MD

6. Achee N, Grieco J. 2012. Is it time to formally recognize spatial repellency for disease prevention? *Outlooks on Pest Management* 23:283-6

7. Achee N, Masuoka P, Smith P, Martin N, Chareonviryiphap T, et al. 2012. Identifying the effective concentration for spatial repellency of the dengue vector *Aedes aegypti. Parasit Vectors* 5:300

8. Achee NL, Bangs MJ, Farlow R, Killeen GF, Lindsay S, et al. 2012. Spatial repellents: from discovery and development to evidence-based validation. *Malar J* 11:164

9. Achee NL, Grieco JP, Andre RG, Rejmankova E, Roberts DR. 2005. A mark-release-recapture study using a novel portable hut design to define the flight behavior of *Anopheles darlingi* in Belize, Central America. *J Am Mosq Control Assoc* 21:366-79

10. Achee NL, Grieco JP, Andre RG, Rejmankova E, Roberts DR. 2007. A mark release-recapture study to define the flight behaviors of *Anopheles vestitipennis* and *Anopheles albimanus* in Belize, Central America. *J Am Mosq Control Assoc* 23:276-82

11. Achee NL, Grieco JP, Rejmankova E, Andre RG, Vanzie E, et al. 2006. Biting patterns and seasonal densities of *Anopheles* mosquitoes in the Cayo District, Belize, Central America with emphasis on *Anopheles darlingi. J Vector Ecol* 31:45-57

12. Achee NL, Korves CT, Bangs MJ, Rejmankova E, Lege M, et al. 2000. *Plasmodium vivax* polymorphs and *Plasmodium falciparum* circumsporozoite proteins in *Anopheles* (Diptera: Culicidae) from Belize, Central America. *J Vector Ecol* 25:203-11

13. Achee NL, Sardelis MR, Dusfour I, Chauhan KR, Grieco JP. 2009. Characterization of spatial repellent, contact irritant, and toxicant chemical actions of standard vector control compounds. *J Am Mosq Control Assoc* 25:156-67

14. Afrane YA, Githeko AK, Yan G. 2012. The ecology of *Anopheles* mosquitoes under climate change: case studies from the effects of deforestation in East African highlands. *Ann N Y Acad Sci* 1249:204-10

15. Alonso PL, Brown G, Arevalo-Herrera M, Binka F, Chitnis C, et al. 2011. A research agenda to underpin malaria eradication. *PLoS Med* 8:e1000406

16. Araujo AP, Araujo Diniz DF, Helvecio E, de Barros RA, de Oliveira CM, et al. 2013. The susceptibility of *Aedes aegypti* populations displaying temephos resistance to *Bacillus thuringiensis* israelensis: a basis for management. *Parasit Vectors* 6:297

17. Back AT, Lundkvist A. 2013. Dengue viruses - an overview. *Infect Ecol Epidemiol* 3

18. Ballenger-Browning KK, Elder JP. 2009. Multi-modal *Aedes aegypti* mosquito reduction interventions and dengue fever prevention. *Trop Med Int Health* 14:1542-51

19. Bangs MJ. 1999. *The susceptibility and behavioral responses of* Anopheles albimanus *Weidemann and* Anopheles vestitipennis *Dyar and Knab (Diptera: Culicidae) to insecticides in northern Belize.* Uniformed Serviced University of the Health Sciences, Bethesda, MD

20. Bautista K. 2012. Vector Control Workshop: Opening Remarks. San Ignacio Town, Cayo District, Belize: Ministry of Health

21. Biogents. 2013. *BG-Sentinel Professional Mosquito Trap.* http://www.bg-sentinel.com/

22. Bohbot JD, Dickens JC. 2012. Odorant receptor modulation: ternary paradigm for mode of action of insect repellents. *Neuropharmacology* 62:2086-95

23. Bohbot JD, Fu L, Le TC, Chauhan KR, Cantrell CL, Dickens JC. 2011. Multiple activities of insect repellents on odorant receptors in mosquitoes. *Med Vet Entomol* 25:436-44

24. Brogdon W, Chan A. 2013. *Guidline for Evaluating Insecticide Resistance in Vectors Using the CDC Bottle Bioassay.* Atlanta, GA: US Centers for Disease Control and Prevention

25. Brown AW, Haworth J, Zahar AR. 1976. Malaria eradication and control from a global standpoint. *J Med Entomol* 13:1-25

26. Campbell-Lendrum D, Molyneux D, Amerasinghe F, Davies C, Fletcher E, et al. 2005. Ecosystems and Vector-borne Disease Control. In *Ecosystems and Human Well-being: Policy Responses*, ed. P Epstein, A Githeko, J Rabinovich, P Weinstein. 3. Washington, DC: Island Press

27. CDC. 2011. Dengue and the *Aedes aegypti* mosquito. San Juan, PR: Dengue Branch, Centers for Disease Control and Prevention

28. CDC. 2014. *Diseases and Conditions*. http://www.cdc.gov/features/diseasesconditions.html

29. Chareonviriyaphap T, Bangs MJ, Suwonkerd W, Kongmee M, Corbel V, Ngoen-Klan R. 2013. Review of insecticide resistance and behavioral avoidance of vectors of human diseases in Thailand. *Parasit Vectors* 6:280

30. Chen CD, Nazni WA, Lee HL, Norma-Rashid Y, Lardizabal ML, Sofian-Azirun M. 2013. Temephos resistance in field *Aedes (Stegomyia) albopictus* (Skuse) from Selangor, Malaysia. *Trop Biomed* 30:220-30

31. Cherington E, Ek E, Cho P, Burgess F, Hernandez B, et al. 2010. Forest Cover and Deforestation in Belize: 1980-2010. In *SERVIR Mesoamerica*. Huntsville, AL: Regional Visualization & Monitoring System (SERVIR)

32. Clark GG, Fernandez-Salas I. 2013. Mosquito vector biology and control in Latin America--a 23rd symposium. *J Am Mosq Control Assoc* 29:251-69

33. Clemons A, Haugen M, Flannery E, Tomchaney M, Kast K, et al. 2010. *Aedes aegypti*: an Emerging Model for Vector Mosquito Development. *Cold Spring Harb Protoc* 2010:pdb emo141

34. Cohnstaedt LW, Allan SA. 2011. Effects of sublethal pyrethroid exposure on the host-seeking behavior of female mosquitoes. *J Vector Ecol* 36:395-403

35. Collier BW, Perich MJ, Boquin GJ, Harrington SR, Francis MJ. 2006. Field evaluation of mosquito control devices in southern Louisiana. *J Am Mosq Control Assoc* 22:444-50

36. Collins FH, Paskewitz SM. 1995. Malaria: current and future prospects for control. *Annu Rev Entomol* 40:195-219

37. Conn J, Quinones M, Povoa M. 2013. Phylogeography, Vectors, and Transmission in Latin America. In *Anopheles Mosquitoes - New Insights Into Malaria Vectors*, ed. S Manguin. Rijeka, Croatia: InTech

38. Cook SM, Khan ZR, Pickett JA. 2007. The use of push-pull strategies in integrated pest management. *Annu Rev Entomol* 52:375-400

39. Curtis CF. 2002. Disease vector control: Development and implementation of control methods. In *Insect Vectors and Human Health*:50-66. Geneva: WHO

40. Curtis CF, Mnzava AE. 2000. Comparison of house spraying and insecticide-treated nets for malaria control. *Bull World Health Organ* 78:1389-400

41. David JP, Ismail HM, Chandor-Proust A, Paine MJ. 2013. Role of cytochrome P450s in insecticide resistance: impact on the control of mosquito-borne diseases and use of insecticides on Earth. *Philos Trans R Soc Lond B Biol Sci* 368:20120429

42. Debboun M, Frances SP, Strickman D. 2007. *Insect Repellents: Principles, Methods and Uses*. Boca Raton, FL: CRC Press

43. Debboun M, Strickman D. 2013. Insect repellents and associated personal protection for a reduction in human disease. *Med Vet Entomol* 27:1-9

44. DeGennaro M, McBride CS, Seeholzer L, Nakagawa T, Dennis EJ, et al. 2013. *orco* mutant mosquitoes lose strong preference for humans and are not repelled by volatile DEET. *Nature* 498:487-91

45. Ditzen M, Pellegrino M, Vosshall LB. 2008. Insect odorant receptors are molecular targets of the insect repellent DEET. *Science* 319:1838-42

46. Durden LA, Mullen GR. 2009. *Medical and Veterinary Entomology*. Amsterdam: Academic Press

47. Enayati A, Hemingway J. 2010. Malaria management: past, present, and future. *Annu Rev Entomol* 55:569-91

48. Endura S. 2012. Technical Data Sheet: Transfluthrin. EF Chemicals: Endura SpA

49. Enserink M. 2002. What mosquitoes want: secrets of host attraction. *Science* 298:90-2

50. Esu E, Lenhart A, Smith L, Horstick O. 2010. Effectiveness of peridomestic space spraying with insecticide on dengue transmission; systematic review. *Trop Med Int Health* 15:619-31

51. Farrar J, Focks D, Gubler D, Barrera R, Guzman MG, et al. 2007. Editorial: Towards a global dengue research agenda. *Tropical Medicine & International Health* 12:695-9

52. Feachem R, Sabot O. 2008. A new global malaria eradication strategy. *Lancet* 371:1633-35

53. Frances SP, Waterson DG, Beebe NW, Cooper RD. 2004. Field evaluation of repellent formulations containing deet and picaridin against mosquitoes in Northern Territory, Australia. *J Med Entomol* 41:414-7

54. Gabaldon A. 1978. What can and cannot be achieved with conventional anti-malaria measures. *Am J Trop Med Hyg* 27:653-8

55. Gaffigan TV, Wilkerson RC, Pecor JE, Stoffer JA, Anderson T. 2012. *Systemic Catalog of Culicidae*. http://www.mosquitocatalog.org/

56. Gallup JL, Sachs JD. 2000. The Economic Burden of Malaria. In *Working Papers: Center for International Development at Harvard University*. Boston, MA

57. Gilles HM. 2002. Historical Outline. In *Essential Malariology*, ed. DA Warell, HM Gilles:3-7. London: Arnold

58. Gonquez D. 2011. *Climate Summary*. http://www.hydromet.gov.bz/Climate_Summary

59. Gravitz L. 2012. Vector control: The last bite. *Nature* 484:S26-7

60. Grieco J. 2000. *The bionomics and vector competence of* Anopheles albimanus *and* Anopheles vestitipennis *in southern Belize, Central America*. Uniformed Services University of the Health Sciences, Bethesda, MD

61. Grieco JP, Achee NL, Andre RG, Roberts DR. 2000. A comparison study of house entering and exiting behavior of *Anopheles vestitipennis* (Diptera: Culicidae) using experimental huts sprayed with DDT or deltamethrin in the southern district of Toledo, Belize, C.A. *J Vector Ecol* 25:62-73

62. Grieco JP, Achee NL, Andre RG, Roberts DR. 2002. Host feeding preferences of *Anopheles* species collected by manual aspiration, mechanical aspiration, and from a vehicle-mounted trap in the Toledo District, Belize, Central America. *J Am Mosq Control Assoc* 18:307-15

63. Grieco JP, Achee NL, Chareonviriyaphap T, Suwonkerd W, Chauhan K, et al. 2007. A new classification system for the actions of IRS chemicals traditionally used for malaria control. *PLoS One* 2:e716

64. Grieco JP, Achee NL, Roberts DR, Andre RG. 2005. Comparative susceptibility of three species of *Anopheles* from Belize, Central America, to *Plasmodium falciparum* (NF-54). *J Am Mosq Control Assoc* 21:279-90

65. Grieco JP, Achee NL, Sardelis MR, Chauhan KR, Roberts DR. 2005. A novel high-throughput screening system to evaluate the behavioral response of adult mosquitoes to chemicals. *J Am Mosq Control Assoc* 21:404-11

66. Grieco JP, Vogtsberger RC, Achee NL, Vanzie E, Andre RG, et al. 2005. Evaluation of habitat management strategies for the reduction of malaria vectors in northern Belize. *J Vector Ecol* 30:235-43

67. Guerra CA, Gikandi PW, Tatem AJ, Noor AM, Smith DL, al. e. 2008. The Limits and Intensity of *Plasmodium falciparum* Transmission: Implications for Malaria Control and Elimination Worldwide. *PLoS Med* 5:e38

68. Hakre S. 2003. *The Epidemiology of Malaria in Belize, 1989-1999*. Uniformed Services University of the Health Sciences, Bethesda, MD

69. Hao H, Wei J, Dai J, Du J. 2008. Host-seeking and blood-feeding behavior of *Aedes albopictus* (Diptera: Culicidae) exposed to vapors of geraniol, citral, citronellal, eugenol, or anisaldehyde. *J Med Entomol* 45:533-9

70. Hemingway J. 2014. The role of vector control in stopping the transmission of malaria: threats and opportunities. *Philos Trans R Soc Lond B Biol Sci* 369:20130431

71. Henderson JP, Westwood R, Galloway T. 2006. An assessment of the effectiveness of the Mosquito Magnet Pro Model for suppression of nuisance mosquitoes. *J Am Mosq Control Assoc* 22:401-7

72. Herrera A. 2002. Mesoamerican Biological Corridor Project: Belize National Report. *Technical Report*, Ministry of Natural Resources and the Environment, Belmopan, Belize

73. Hill CA, Kafatos FC, Stansfield SK, Collins FH. 2005. Arthropod-borne diseases: vector control in the genomics era. *Nat Rev Microbiol* 3:262-8

74. Hiwat H, De Rijk M, Andriessen R, Koenraadt CJ, Takken W. 2011. Evaluation of methods for sampling the malaria vector *Anopheles darlingi* (Diptera, Culicidae) in Suriname and the relation with its biting behavior. *J Med Entomol* 48:1039-46

75. Horstick O, Runge-Ranzinger S, Nathan MB, Kroeger A. 2010. Dengue vector-control services: how do they work? A systematic literature review and country case studies. *Trans R Soc Trop Med Hyg* 104:379-86

76. IMF. 2013. *Report for Selected Countries and Subjects*. www.imf.org/external/pubs/ft/weo/2013/01/weodata

77. IRAC. 2014. *Insecticide Resistance Management for Vector Control*. http://www.irac-online.org/documents/public-health-irm-poster/?ext=pdf

78. Iwashita H, Dida GO, Sonye GO, Sunahara T, Futami K, et al. 2014. Push by a net, pull by a cow: can zooprophylaxis enhance the impact of insecticide treated bed nets on malaria control? *Parasit Vectors* 7:52

79. Kain P, Boyle SM, Tharadra SK, Guda T, Pham C, et al. 2013. Odour receptors and neurons for DEET and new insect repellents. *Nature* 502:507-12

80. Karunamoorthi K. 2011. Vector control: a cornerstone in the malaria elimination campaign. *Clin Microbiol Infect* 17:1608-16

81. Kawada H, Ohashi K, Dida GO, Sonye G, Njenga SM, et al. 2014. Insecticidal and repellent activities of pyrethroids to the three major pyrethroid-resistant malaria vectors in western Kenya. *Parasit Vectors* 7:208

82. Kitau J, Pates H, Rwegoshora TR, Rwegoshora D, Matowo J, et al. 2010. The effect of Mosquito Magnet Liberty Plus trap on the human mosquito biting rate under semi-field conditions. *J Am Mosq Control Assoc* 26:287-94

83. Kline DL. 2006. Traps and trapping techniques for adult mosquito control. *J Am Mosq Control Assoc* 22:490-6

84. Mabaso ML, Sharp B, Lengeler C. 2004. Historical review of malarial control in southern African with emphasis on the use of indoor residual house-spraying. *Trop Med Int Health* 9:846-56

85. malERA. 2011. A research agenda for malaria eradication: vector control. *PLoS Med* 8:e1000401

86. Manda H, Arce LM, Foggie T, Shah P, Grieco JP, Achee NL. 2011. Effects of irritant chemicals on *Aedes aegypti* resting behavior: is there a simple shift to untreated "safe sites"? *PLoS Negl Trop Dis* 5:e1243

87. Manda H, Shah P, Polsomboon S, Chareonviriyaphap T, Castro-Llanos F, et al. 2013. Contact irritant responses of *Aedes aegypti* Using sublethal concentration and focal application of pyrethroid chemicals. *PLoS Negl Trop Dis* 7:e2074

88. Matthews G. 2011. *Integrated Vector Management: Controlling Vectors of Malaria and Other Insect Vector Borne Diseases*. West Sussex, UK: John Wiley & Sons. 234 pp.

89. Meier M. 2011. Technical Grade Transfluthrin Spatial Repellency Prootocol. ed. N Achee. Email

90. Menger DJ, Otieno B, de Rijk M, Mukabana WR, van Loon JJ, Takken W. 2014. A push-pull system to reduce house entry of malaria mosquitoes. *Malar J* 13:119

91. Mills A, Lubell Y, Hanson K. 2008. Malaria eradication: the economic, financial and institutional challenge. *Malar J* 7 Suppl 1:S11

92. Missawa NA, Ribeiro AL, Maciel GB, Zeilhofer P. 2011. Comparison of capture methods for the diagnosis of adult anopheline populations from State of Mato Grosso, Brazil. *Rev Soc Bras Med Trop* 44:555-60

93. Mohammed H, Smith J. 2011. First record of *Anopheles albimanus* from St Kitts. *West Indian Med J* 60:562-3

94. Moore SJ, Mordue Luntz AJ, Logan JG. 2012. Insect bite prevention. *Infect Dis Clin North Am* 26:655-73

95. Muirhead-Thomson RC. 1950. DDT and gammexane as residual insecticides against *Anopheles gambiae* in African houses. *Trans R Soc Trop Med Hyg* 43:401-12

96. Murray KO, Rodriguez LF, Herrington E, Kharat V, Vasilakis N, et al. 2013. Identification of dengue fever cases in Houston, Texas, with evidence of autochthonous transmission between 2003 and 2005. *Vector Borne Zoonotic Dis* 13:835-45

97. Mwangangi JM, Mbogo CM, Orindi BO, Muturi EJ, Midega JT, et al. 2013. Shifts in malaria vector species composition and transmission dynamics along the Kenyan coast over the past 20 years. *Malar J* 12:13

98. Nabarro DN, Tayler EM. 1998. Global Health: The 'roll back malaria' campaign. *Science* 280:2067-68

99. Najera JA, Gonzalez-Silva M, Alonso PL. 2011. Some lessons for the future from the Global Malaria Eradication Programme (1955-1969). *PLoS Med* 8:e1000412

100. Ndiath MO, Mazenot C, Sokhna C, Trape JF. 2014. How the malaria vector *Anopheles gambiae* adapts to the use of insecticide-treated nets by African populations. *PLoS One* 9:e97700

101. Njiru BN, Mukabana WR, Takken W, Knols BG. 2006. Trapping of the malaria vector *Anopheles gambiae* with odour-baited MM-X traps in semi-field conditions in western Kenya. *Malar J* 5:39

102. Nkya TE, Akhouayri I, Kisinza W, David JP. 2013. Impact of environment on mosquito response to pyrethroid insecticides: facts, evidences and prospects. *Insect Biochem Mol Biol* 43:407-16

103. NRC. 2008. *Vector-Borne Diseases: Understanding the Environmental, Human Health, and Ecological Connections, Workshop Summary (Forum on Microbial Threats)*. Washington, DC: The National Academies Press

104. Obenauer PJ, Abdel-Dayem MS, Stoops CA, Villinski JT, Tageldin R, et al. 2013. Field responses of *Anopheles gambiae* complex (Diptera: Culicidae) in Liberia using yeast-generated carbon dioxide and synthetic lure-baited light traps. *J Med Entomol* 50:863-70

105. Ogoma SB, Lorenz LM, Ngonyani H, Sangusangu R, Kitumbukile M, et al. 2014. An experimental hut study to quantify the effect of DDT and airborne pyrethroids on entomological parameters of malaria transmission. *Malar J* 13:131

106. Ogoma SB, Moore SJ, Maia MF. 2012. A systematic review of mosquito coils and passive emanators: defining recommendations for spatial repellency testing methodologies. *Parasit Vectors* 5:287

107. Ogoma SB, Ngonyani H, Simfukwe ET, Mseka A, Moore J, Killeen GF. 2012. Spatial repellency of transfluthrin-treated hessian strips against laboratory-reared *Anopheles arabiensis* mosquitoes in a semi-field tunnel cage. *Parasit Vectors* 5:54

108. PAHO. 1994. Status of malaria programs in the Americas, Pan American Health Organization, Washington, DC

109. PAHO. 1998. Plan Continental de ampliación e intensificación del combate a *Aedes aegypti*. *Revista Panamericana de Salud Pública* 3:124-30

110. Pates HV, Line JD, Keto AJ, Miller JE. 2002. Personal protection against mosquitoes in Dar es Salaam, Tanzania, by using a kerosene oil lamp to vaporize transfluthrin. *Med Vet Entomol* 16:277-84

111. Paz-Soldan VA, Plasai V, Morrison AC, Rios-Lopez EJ, Guedez-Gonzales S, et al. 2011. Initial assessment of the acceptability of a Push-Pull *Aedes aegypti* control strategy in Iquitos, Peru and Kanchanaburi, Thailand. *Am J Trop Med Hyg* 84:208-17

112. Pellegrino M, Steinbach N, Stensmyr MC, Hansson BS, Vosshall LB. 2011. A natural polymorphism alters odour and DEET sensitivity in an insect odorant receptor. *Nature* 478:511-4

113. Pickett JA, Birkett MA, Logan JG. 2008. DEET repels ORNery mosquitoes. *Proc Natl Acad Sci U S A* 105:13195-6

114. Pyke B, Rice M, Sabine B, Zalucki MP. 1987. The push-pull strategy - behavioural control of *Heliothis*. *Austr. Cotton Grow.* May-July:7-9

115. Ramsey JM, Salinas E, Bown DN, Rodriguez MH. 1994. Plasmodium vivax sporozoite rates from *Anopheles albimanus* in southern Chiapas, Mexico. *J Parasitol* 80:489-93

116. Ranson H, N'Guessan R, Lines J, Moiroux N, Nkuni Z, Corbel V. 2011. Pyrethroid resistance in African anopheline mosquitoes: what are the implications for malaria control? *Trends Parasitol* 27:91-8

117. Reddy GV, Guerrero A. 2010. New pheromones and insect control strategies. *Vitam Horm* 83:493-519

118. Rejmankova E, Pope KO, Roberts DR, Lege MG, Andre R, et al. 1998. Characterization and detection of *Anopheles vestitipennis* and *Anopheles punctimacula* (Diptera: Culicidae) larval habitats in Belize with field survey and SPOT satellite imagery. *J Vector Ecol* 23:74-88

119. Rejmankova E, Roberts D, Harbach R, Pecor J, Peyton E, et al. 1993. Environmental and Regional Determinants of *Anopheles* (Diptera:Culicidae) Larval Distribution in Belize, Central America. *Journal of Environmental Entomology* 22:978 - 92

120. Rejmankova E, Roberts DR, Manguin S, Pope KO, Komarek J, Post RA. 1996. *Anopheles albimanus* (Diptera: Culicidae) and cyanobacteria: an example of larval habitat selection. *Environ Entomol* 25:1058-67

121. Revay EE, Kline DL, Xue RD, Qualls WA, Bernier UR, et al. 2013. Reduction of mosquito biting-pressure: spatial repellents or mosquito traps? A field comparison of seven commercially available products in Israel. *Acta Trop* 127:63-8

122. Roberts D. 1993. Insecticide Repellency in Malaria Vector Control: A Position Paper. *Rep. 81131*, Medical Service Corporation Internaitonal/USAID, Arlington, VA

123. Roberts D, Tren R, Bate R, Zambone J. 2010. *The Excellent Powder: DDT's Political and Scientific History*. Indianapolis, IN: Dog Ear Publishing

124. Roberts DR, Alecrim WD, Hshieh P, Grieco JP, Bangs M, et al. 2000. A probability model of vector behavior: effects of DDT repellency, irritancy, and toxicity in malaria control. *J Vector Ecol* 25:48-61

125. Roberts DR, Andre RG. 1994. Insecticide resistance issues in vector-borne disease control. *Am J Trop Med Hyg* 50:21-34

126. Roberts DR, Manguin S, Mouchet J. 2000. DDT house spraying and re-emerging malaria. *Lancet* 356:330-2

127. Roberts DR, Manguin S, Rejmankova E, Andre R, Harbach RE, et al. 2002. Spatial distribution of adult *Anopheles darlingi* and *Anopheles albimanus* in relation to riparian habitats in Belize, Central America. *J Vector Ecol* 27:21-30

128. Roberts DR, Vanzie E, Bangs MJ, Grieco JP, Lenares H, et al. 2002. Role of residual spraying for malaria control in Belize. *J Vector Ecol* 27:63-9

129. Rutledge LC, Gupta RK, Piper GN, Lowe CA. 1994. Studies on the inheritance of repellent tolerances in *Aedes aegypti*. *J Am Mosq Control Assoc* 10:93-100

130. Sachs P, Diaz Rodriguez GA, Briceno I, King R, Achee NL, Grieco JP. 2013. Comparison of experimental hut entrance and exit behavior between *Anopheles*

darlingi from the Cayo District, Belize, and Zungarococha, Peru. *J Am Mosq Control Assoc* 29:319-27

131. Salazar FV, Achee NL, Grieco JP, Prabaripai A, Eisen L, et al. 2012. Evaluation of a peridomestic mosquito trap for integration into an *Aedes aegypti* (Diptera: Culicidae) push-pull control strategy. *J Vector Ecol* 37:8-19

132. Salazar FV, Achee NL, Grieco JP, Prabaripai A, Ojo TA, et al. 2013. Effect of *Aedes aegypti* exposure to spatial repellent chemicals on BG-SentinelTM trap catches. *Parasit Vectors* 6:145

133. Schmied WH, Takken W, Killeen GF, Knols BG, Smallegange RC. 2008. Evaluation of two counterflow traps for testing behaviour-mediating compounds for the malaria vector *Anopheles gambiae* s.s. under semi-field conditions in Tanzania. *Malar J* 7:230

134. Siegert PY, Walker E, Miller JR. 2009. Differential behavioral responses of *Anopheles gambiae* (Diptera: Culicidae) modulate mortality caused by pyrethroid-treated bednets. *J Econ Entomol* 102:2061-71

135. Sikaala CH, Killeen GF, Chanda J, Chinula D, Miller JM, et al. 2013. Evaluation of alternative mosquito sampling methods for malaria vectors in Lowland South--East Zambia. *Parasit Vectors* 6:91

136. Silver JB. 2008. *Mosquito Ecology: Field Sampling Methods*. Dordrecht, The Netherlands: Springer

137. Sinka ME. 2013. Global Distribution of the Dominant Vector Species of Malaria. In *Anopheles Mosquitoes - New Insights Into Malaria Vectors*, ed. S Manguin. Rijeka, Croatia: InTech

138. Smith JP, Cope EH, Walsh JD, Hendrickson CD. 2010. Ineffectiveness of mass trapping for mosquito control in St. Andrews State Park, Panama City Beach, Florida. *J Am Mosq Control Assoc* 26:43-9

139. Snow RW, Guerra CA, Noor AM, Myint HY, Hay SI. 2005. The global distribution of clinical episodes of *Plasmodium falciparum* malaria. *Nature* 434:214-7

140. Soderlund DM. 2008. Pyrethroids, knockdown resistance and sodium channels. *Pest Manag Sci* 64:610-6

141. Soderlund DM, Clark JM, Sheets LP, Mullin LS, Piccirillo VJ, et al. 2002. Mechanisms of pyrethroid neurotoxicity: implications for cumulative risk assessment. *Toxicology* 171:3-59

142. Stahl HC, Butenschoen VM, Tran HT, Gozzer E, Skewes R, et al. 2013. Cost of dengue outbreaks: literature review and country case studies. *BMC Public Health* 13:1048

143. Stanczyk NM, Brookfield JF, Field LM, Logan JG. 2013. *Aedes aegypti* mosquitoes exhibit decreased repellency by DEET following previous exposure. *PLoS One* 8:e54438

144. Stanczyk NM, Brookfield JF, Ignell R, Logan JG, Field LM. 2010. Behavioral insensitivity to DEET in *Aedes aegypti* is a genetically determined trait residing in changes in sensillum function. *Proc Natl Acad Sci U S A* 107:8575-80

145. Syed Z, Leal WS. 2008. Mosquitoes smell and avoid the insect repellent DEET. *Proc Natl Acad Sci U S A* 105:13598-603

146. Tainchum K, Polsomboon S, Grieco JP, Suwonkerd W, Prabaripai A, et al. 2013. Comparison of *Aedes aegypti* (Diptera: Culicidae) resting behavior on two fabric types under consideration for insecticide treatment in a push-pull strategy. *J Med Entomol* 50:59-68

147. Takken W. 2010. Push-pull strategies for vector control. *Malaria Journal* 9 (Suppl 2):I16

148. Tanner M, de Savigny D. 2008. Malaria eradication back on the table. *Bull World Health Organ* 86:82

149. Townson H, Nathan MB, Zaim M, Guillet P, Manga L, et al. 2005. Exploiting the potential of vector control for disease prevention. *Bull World Health Organ* 83:942-7

150. Tsitsanou KE, Thireou T, Drakou CE, Koussis K, Keramioti MV, et al. 2012. *Anopheles gambiae* odorant binding protein crystal complex with the synthetic repellent DEET: implications for structure-based design of novel mosquito repellents. *Cell Mol Life Sci* 69:283-97

151. UNDP. 2013. Human Development Report 2013 Rise of the South: Human Progress in a Diverse World, United Nations, Geneva

152. Vanzie E. 2008. Assessment of the Impact of Climate Change on Belize's Health Sector: Dengue and Dengue Hemorrhagic Fever, Ministry of Health, Belize, Belmopan, Belize

153. Vasilakis N, Weaver SC. 2008. The history and evolution of human dengue emergence. *Adv Virus Res* 72:1-76

154. von Witzendorff C, Matthes HF, Luciuc R, Reich B, Kalinna B. 2004. A new lure for host-seeking anthropophilic mosquitoes and a novel type of a simple, non-

CO2 mosquito trap. *International Journal of Medical Microbiology* 293 (Suppl. 38):50

155. Wagman J, Grieco J, King R, Briceno I, Bautista K, et al. 2013. First Record and Demonstration of a Southward Expansion of *Aedes albopictus* into Orange Walk Town, Belize, Central America. *Journal of the American Mosquito Control Association* 29:380-2

156. Warrell DA, Gilles HM. 2002. *Essential Malariology*. London: Arnold

157. Weaver SC, Reisen WK. 2010. Present and future arboviral threats. *Antiviral Res* 85:328-45

158. WHO. 1975. *Manual on Practical Entomology in Malaria Part II: Methods and Techniques*. Geneva: World Health Organization

159. WHO. 2003. Insect Vectors and Human Health, World Health Organization, Geneva, Switzerland

160. WHO. 2004. World Health Report 2004: Changing History, World Health Organization, Geneva

161. WHO. 2006. Indoor Resisual Spraying: Use of indoor residual spraying for scaling up global malaria control and elimination. *Position Paper*, World Health Organization, Geneva

162. WHO. 2006. *Malaria vector control and personal protection*. Geneva: WHO Press

163. WHO. 2006. *Pesticides and their application for the control of vectors and pests of public health importance*. pp 125. Geneva: WHO Press

164. WHO. 2007. WHO recommended insecticides products for treatment of mosquito nets for malaria vector control. ed. WHOPES. Geneva: WHO Press

165. WHO. 2009. *Dengue: guidelines for diagnosis, treatment, prevention and control - New edition*. World Health Organizaion Special Programme for Research and Training in Tropical Diseases

166. WHO. 2009. WHO recommended insecticides for indoor residual spraying against malaria vectors. ed. WHOPES. Geneva: WHO Press

167. WHO. 2010. Innovative vector control interventions - 2009 annual report, World Health Organization, Geneva

168. WHO. 2012. *Handbook for integrated vector management*. Geneva: WHO Press

169. WHO. 2012. *World Malaria Report 2012*. Geneva, Switzerland: WHO Press

170. WHO. 2013. *Training Module on Malaria Control: Entomology and Vector Control*. Geneva, Switzerland: WHO Press

171. WHO. 2014. *Health Topics*. http://www.who.int/topics/en/

172. WHOPES. 2013. *Guidelines for Efficacy Testing of Spatial Repellents*. Geneva, Switzerland: World Health Organization

173. Wilkerson RC, Strickman D, Litwak TR. 1990. Illustrated key to the female anopheline mosquitoes of Central America and Mexico. *J Am Mosq Control Assoc* 6:7-34

174. Wong J, Bayoh N, Olang G, Killeen GF, Hamel MJ, et al. 2013. Standardizing operational vector sampling techniques for measuring malaria transmission intensity: evaluation of six mosquito collection methods in western Kenya. *Malar J* 12:143

175. WRBU. 2013. *Medically Important Mosquitoes*. http://wrbu.si.edu/index.html