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**The Effects of Pre-exposure to DEET on the Downstream Blood-Feeding Behaviors of
Aedes aegypti Mosquitoes¹**

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Abstract

Mosquito behavior is heavily influenced by the chemical molecules in the environment. Modifying insect behavior by harnessing this knowledge to reduce vector-host contact is a powerful method for disease prevention. N,N-Diethyl-meta-toluamide (DEET) is the most widely used insect repellent on the market and an excellent example of a chemical that has been used to modify insect behavior for disease prevention. However, genetic insensitivity and habituation in *Aedes aegypti* (L.) mosquitoes after pre-exposure to DEET have been reported. In this study, we investigated the effect of pre-exposure to DEET on the downstream blood-feeding behavior of *Ae. aegypti* mosquitoes and the duration of the effect. We exposed mosquitoes to four different DEET concentrations: 0.10, 0.12, 0.14, and 0.16% for 10 min then allowed the mosquitoes to blood-feed on an artificial blood-feeding system either immediately or after being held for 1, 3, 6, or 24 h following DEET exposure. We found that pre-exposing *Ae. aegypti* mosquitoes to 0.14 or 0.16% DEET lowered their blood engorgement level, but did not alter their landing and probing behavior when compared to the control test populations. The reduction in complete blood-feeding was observed at all time periods tested, but was only statistically significant at 3 and 6 h after the pre-exposure process. Future studies analyzing the effect of this behavior using arbovirus-infected mosquitoes are needed to address the concern of potentially increased vectorial capacity.

Keywords: *Aedes aegypti*, DEET, behavior, blood-feeding

Mosquito behavior is heavily influenced by chemical molecules in the environment that either attract or repel mosquitoes. The maxillary palps and antennae are the main sensory organs of the mosquito and contain abundant sensilla which house the odorant receptor neurons (Amer

49 and Mehlhorn 2006, Hallem et al. 2006, Paluch et al. 2010). The insect olfactory process starts
50 when odorant molecules enter the pores located on the sensilla. Each sensillum contains
51 olfactory receptor neurons that have odorant receptors on its surface. As the molecule enters the
52 pores, the odorant binding protein binds and solubilizes the molecule to be transported to the
53 dendrite of the neuron. When the odorant molecule is recognized by the appropriate odorant
54 receptor, the neuron becomes activated and transfers the information to one of the glomeruli in
55 the antennal lobe of the brain that then further delivers the signal to the brain mushroom body,
56 which processes learning and memory, and brain lateral horn, where the information directs
57 innate behaviors (Guidobaldi et al. 2014, Suh et al. 2014, Twick et al. 2014).

58 As insect bites are not only a source of nuisance but also can spread disease causing
59 organisms, the World Health Organization stated that reducing the contact between vectors and
60 human hosts is a very powerful method to prevent infection (WHO 2009). Vector control
61 chemicals possess three different modes of action: repellent (deter biting without direct contact),
62 irritant (deter biting, but requires contact between vector and treated surface), and toxicant (kills
63 the vector) (Chareonviriyaphap et al. 2004, Grieco et al. 2007). The use of topical insect
64 repellent is a preventive measure that is widely available and used to reduce vector biting (Gupta
65 and Rutledge 1994, CDC 2015). N,N-Diethyl-meta-toluamide (DEET) is an example of a topical
66 insect repellent that has been on the market for almost 70 years. Due to its cost, effectiveness,
67 and safety, DEET is one of the most widely used insect repellents available on the market
68 (Weeks et al. 2012). In addition to its repellent activity, DEET also has been demonstrated to
69 have irritant and toxic effects (Licciardi et al. 2006). Centers for Disease Control and Prevention
70 guidelines for DEET application mentioned that concentration <10% confers 1-2 h protection

71 and the efficacy plateaus after 50% (CDC 2015). Longer protection can be achieved by using
72 different DEET formulations or chemical carriers (Kalyanasundaram and Mathew 2006).

73 Unfortunately, insect insensitivity to DEET has been reported (Stanczyk et al. 2010,
74 Pellegrino et al. 2011). Previous studies have shown that insensitivity to DEET seemed to be a
75 genetic trait (Stanczyk et al. 2010, Pellegrino et al. 2011). One study showed that even though
76 there is a genetic determinant that causes insensitivity to DEET, *Aedes aegypti* (L.) mosquitoes
77 can also “learn” to avoid DEET from initial exposure and becomes less sensitive upon
78 subsequent exposures (Stanczyk et al. 2013). The study showed that mosquitoes that have
79 previously been exposed to 20% DEET, regardless of whether the DEET was applied to a human
80 arm or only a heat source, would become less sensitive when they were exposed to DEET 3 h
81 after the initial exposure. Another study, that included more odorants, showed similar results and
82 found that habituation did not occur with all chemicals tested (Vinauger et al. 2014). These
83 studies demonstrate that previous exposure to DEET may alter the behavior of exposed
84 mosquitoes.

85 Because of the relatively long protection that can be provided by DEET and the non-
86 uniformity of DEET application in the population, it is important to take a step back and observe
87 if pre-exposure to DEET can alter the most important aspect of mosquito behavior - their blood-
88 feeding behavior. Our study aim was to assess the effect of DEET pre-exposure on the landing
89 and probing behaviors and the engorgement levels of *Aedes aegypti* mosquitoes after pre-
90 exposure to DEET.

91 **Materials and Methods**

92 **Mosquito rearing.** *Aedes aegypti* mosquitoes (Belize strain $\leq$ F5) were reared according
93 to standard laboratory procedures. The colony was derived from wild-caught larvae (P1) in

Orange Walk Town, Belize (18°04.938'N, 88°33.390'W) in 2014. Egg strips were soaked in water and vacuumed for 1 h to help synchronize the hatching process. The larvae were fed with Cichlid Gold™ fish food (Hikari USA, Hayward, CA). All mosquitoes were maintained at 28°C with 80% humidity and 12 h light-dark cycle. After molting into second instar larvae they are separated into groups of 50 larvae in individual 450 ml plastic cups. After approximately 7 d, pupae were manually sorted into groups of 250 female and placed in 3.9-liter bucket cages (white plastic bucket with attached sleeve for mosquito collection access). At 4-5 d post eclosion, adult female mosquitoes were provided with 10% sugar solution (Duncraft Inc., Concord, NH) in water from soaked cotton balls and were starved for 24 h before the assay. For assays where the mosquitoes were held for 24 h after exposure to DEET, they were provided with a water soaked cotton pad.

Exposure assay. DEET pre-exposure. Thirty female *Ae. aegypti* mosquitoes were pre-exposed to DEET (treatment cohort) in a high throughput screening system (HITSS) chamber for 10 min and 30 females were pre-exposed to ethanol (control cohort) in another chamber as control for 10 min (Grieco et al. 2005). The chamber is a metal cylinder with a smaller cylinder metal insert where material that had been pretreated with the chemical of choice could be attached to it with magnets. Both ends of the chamber are secured with removable plastic caps equipped with dental dam-gated holes for introducing the mosquitoes into the chambers. The DEET concentrations that were used in this study were 0.10, 0.12, 0.14, and 0.16% diluted in ethanol from 5% DEET stock solution (USDA, Beltsville, MD). Those concentrations were chosen based on the dose-response curve obtained from a dengue-*Ae. aegypti* behavior experiments (VS, unpublished data). An additional experiment using 5% DEET but with only a 1

min exposure was also conducted to assess the effect of a higher concentration of DEET on short-term exposure.

Holding system. We tested five different holding periods: immediately after exposure/no holding time (T0) and after 1 (T1), 3 (T3), 6 (T6), and 24 h (T24) post-exposure (Fig. 1). After pre-exposure, the mosquitoes were collected using a mechanical aspirator and placed into holding tubes (plastic tube with mesh screen on the bottom and open-ended tops that were closed with rubber caps). T0 mosquitoes were immediately used for testing. For the other holding times, the mosquitoes were transferred into pint cups and incubated at 28°C with 80% humidity until tested. Once the holding times were over, the mosquitoes were aspirated from the pint cups into the holding tubes. The holding tubes containing mosquitoes were given to a third party who randomly labeled them in order to reduce bias in data recording by the personnel conducting the experiments.

Post-exposure blood-feeding behavior observation. To observe the blood-feeding behavior, 20 mosquitoes from each test population were put into separate Plexiglas[®] boxes with an artificial blood-feeding system placed on top of the box (Fig. 2). The mosquitoes were allowed to blood-feed for 20 min. The landing, probing, and engorgement behaviors were observed. The observation for landing and probing were conducted at 30 s intervals for the first 5 min. Landing was defined as the number of mosquitoes on the blood source, while probing was defined as the number of mosquitoes probing or feeding at the blood source. At the end of 20 min, the mosquitoes were collected, put in the freezer (-20°C) to knock them down, and then graded for engorgement according to the method by Pilitt and Jones (Pilitt and Jones 1972). The observers were blinded as to the status of the test cohorts. Six replicates were conducted to

138 obtain necessary statistical power with the observers switching the box that they observe after
139 three replicates.

Data analysis. The number of mosquitoes landing and probing were compared between the treatment and control cohorts using the Mann-Whitney U test (SPSS 22.0 software IBM Corp., Armonk, NY) (Mann and Whitney 1947). The corresponding effect size for landing and probing was summarized using the rank-biserial correlation, which measured the strength of association between condition and number of landings/probings. Values close to 0 indicated minimal difference between cohorts, and values close to 1 indicated maximal difference between treatment cohorts. The power of landing and probing experiments depended on the number of replicates tested (N= 6). The blood engorgement level data were combined into two groups: 0-3 (no to little blood-feeding) and 4-5 (engorged) then analyzed with the Fisher's exact test with two-tailed *P*-value using GraphPad (GraphPad Software, La Jolla, CA) and Stepdown-Sidak post hoc analysis on Microsoft Excel 2010 (Microsoft Corp., Albuquerque, NM) (Holm 1979). The power of blood engorgement level depended on the number of mosquitoes used in the experiments (N= 120).

153 **Results**

Landing and probing. The effect of DEET on the landing and probing behaviors of *Ae. aegypti* mosquitoes was determined by comparing the landing and probing rates between mosquitoes that were pre-exposed to DEET (treatment cohort) and ethanol (control cohort) (Table 1). No statistically significant difference was observed between the treatment and control populations at any concentration of DEET used in the pre-exposure at any given incubation time. This suggested that pre-exposure to DEET did not affect landing or probing behavior ($P = 0.10$ for both landing and probing).

161 **Engorgement.** To examine the effect of DEET on blood engorgement levels in *Ae.*
162 *aegypti* mosquitoes, we combined the data for the replicates at each time period for each
163 exposure cohort. We then compared the number of mosquitoes with no to moderate engorgement
164 (grades 0-3) with those that took a nearly complete blood meal (grades 4 and 5) for mosquitoes
165 that were pre-exposed to DEET or ethanol (control) (Table 2). No statistically significant
166 difference was observed when the mosquitoes were given a blood source immediately after
167 DEET exposure process (T0) at any concentration. Therefore, for subsequent experiments, the
168 lowest DEET concentration (0.10%) was not tested anymore. Similarly, when tested after 1 h
169 incubation (T1), the engorgement level of mosquitoes that were pre-exposed to any
170 concentration of DEET did not show any statistical significant difference. Therefore, in the
171 subsequent experiments, 0.12% DEET was also dropped.

172 The engorgement level of mosquitoes that were pre-exposed to 0.14 or 0.16% DEET
173 were reduced when compared to the control after they had been incubated for 3 or 6 h after the
174 pre-exposure step ($P \leq 0.02$). This reduction of blood engorgement level was still detectable at
175 24 h but it was no longer statistically significant ($P = 0.38$). Overall, the blood engorgement level
176 was statistically reduced within 24 h after pre-exposure to 0.14 or 0.16% DEET ($P < 0.01$).

177 **Pre-exposure to high DEET concentration.** As DEET is marketed at higher
178 concentrations than what we tested, we conducted an additional experiment to see if exposure at
179 a higher concentration, but for a shorter time, would produce a similar result as the previous
180 experiments (lower concentration at relatively longer time). All three observed behaviors;
181 landing, probing, and engorgement levels; were not significantly different between mosquitoes
182 pre-exposed to 5% DEET and mosquitoes pre-exposed to ethanol when they were tested 6 h after

they had been exposed for 1 min (landing and probing $P = 0.087$; blood engorgement level $P = 0.088$).

Discussion

We found that pre-exposure to DEET reduced the ability of *Ae. aegypti* mosquitoes to obtain a full blood meal from 3-6 h after their exposure to 0.14% or 0.16% DEET. This may have implications on vectorial capacity. Pre-exposure to certain chemicals has been shown to alter the mosquito's subsequent behavioral responses to the same or other chemicals (Stanczyk et al. 2013, Thany et al. 2015). Our study addressed the possibility changes in blood-feeding behavior in *Ae. aegypti* mosquitoes following DEET exposure.

We found that pre-exposure to DEET may alter certain aspect of *Ae. aegypti* blood-feeding behavior. Mosquitoes that had been pre-exposed to DEET did not show any difference in their landing and probing rates compared to the control cohorts; however, they imbibed less blood than the controls. The significant difference in engorgement level was observed only in the cohorts pre-exposed to 0.14 or 0.16% DEET, and occurred consistently at 3 and 6 h after pre-exposure. This concentration is the minimal necessary concentration required to induce irritancy response in *Ae. aegypti* Liverpool populations (VS, unpublished data). The effect also seemed to be more pronounced as the concentration increased.

In contrast, no change in blood-feeding behavior was observed when the mosquitoes were tested immediately after DEET exposure. While this seems counterintuitive, we speculate that when the mosquitoes were tested immediately, they did not have sufficient time to recover from handling during the pre-exposure process and thus blood-feeding was inhibited in both cohorts, masking the effect of DEET. Moreover, as the incubation period increased up to 6 h post exposure, the mosquitoes became hungrier and fed better on the blood source.

Even though we did not see any changes in the landing and probing rates of the mosquitoes between those pre-exposed to DEET and ethanol, the differences in engorgement levels at the end of the 20-min blood-feeding period suggested that there may have been differences that were not observed. These may have been missed because landing and engorgement behaviors were only observed during the first 5 min of the blood-feeding observation.

The level of DEET that evaporates from or is absorbed by the skin varies greatly depending on the initial concentration applied, how long it has been applied, and the formulation of the DEET solution (Reifenrath et al. 1989, Kalyanasundaram and Mathew 2006, EPA 2015). This existing variation makes it very difficult, if not impossible, to ascertain specific concentrations to be tested. We started our experiments with low DEET concentrations for 10 min based on a previous experiment to find the concentration of DEET that elicited irritancy behavior, which was 0.14% (VS, unpublished data). After we saw that mosquitoes pre-exposed to 0.14 or 0.16% DEET for 10 min displayed significantly less engorgement, we tested a higher concentration of DEET (5%), but for a shorter time (1 min). Interestingly, no behavioral changes were observed in the landing, probing, or engorgement levels in the mosquitoes pre-exposed to 5% DEET for 1 min.

Although mosquitoes pre-exposed to DEET were still at least 3-fold less likely to obtain a complete blood meal at 24 h as those pre-exposed to ethanol, these differences were no longer statistically significant. The lack of statistical significance may be due to the relatively poor feeding in the mosquitoes held for 24 h. In the studies conducted at 24 h, the control mosquitoes were significantly less likely to obtain a complete blood meal than those tested at 3 or 6 h ($P \leq 0.01$), making it more difficult to obtain statistical significance despite the >3-fold difference in

feeding success. The reduced re-feeding might have been because these mosquitoes had been provided with a water source that might have increased their satiety levels.

Aedes aegypti mosquitoes can transmit many arboviruses, such as: dengue, chikungunya, yellow fever, or Zika viruses. If these mosquitoes were infected with an arbovirus and then were repelled by DEET, they might not feed to engorgement on their next host, thus prompting them to bite more often on more hosts, further spreading the virus. Moreover, a previous study also indicated that pre-exposure to DEET reduced the mosquito repellency to the subsequent DEET exposure rendering the repellent to be less effective in protecting against mosquito bites (Stanczyk et al. 2013). In addition, dengue infection in mosquitoes did not alter their behavior against DEET (Frances et al. 2011). Furthermore, it is interesting to see if similar behavioral changes also happen in other disease vectors such as Anopheles mosquito, sand flies, and kissing bugs.

In conclusion, even though DEET has been around for a long time, there are still many aspects of DEET use that still need to be better understood. Additionally, even though there is a growing body of evidence that chemical pre-exposure can alter the subsequent behavior of other insects that are vectors for diseases, the number of research is still lacking. The possibility of DEET pre-exposure causing higher incidence of arboviral infection is of particular concern because DEET application is a part of the greater efforts in reducing vector and host contact to reduce the incidence of the disease. Further research using arbovirus-infected mosquitoes is necessary to address this concern and expanding the scope of similar behavioral alteration research to other diseases vectors may help us better utilize the tools we have in the fight against vector-borne diseases.

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- 327

Table 1. The rank-biserial correlation of landing and probing behaviors between DEET pre-exposed and control mosquitoes.

Incubation time	DEET concentration							
	0.10%		0.12%		0.14%		0.16%	
	Landing	P-value	Landing	P-value	Landing	P-value	Landing	P-value
T0	0.39	0.26	0.22	0.48	0.03	0.90	0.06	0.87
T1	N/A ^a	N/A	0.28	0.42	0.42	0.21	0.56	0.10
T3	N/A	N/A	N/A	N/A	0.36	0.30	0.31	0.38
T6	N/A	N/A	N/A	N/A	0.19	0.57	0.42	0.23
T24	N/A	N/A	N/A	N/A	0.33	0.14	0.33	0.14
Incubation time	Probing		Probing		Probing		Probing	
	P-value		P-value		P-value		P-value	
	Landing	P-value	Landing	P-value	Landing	P-value	Landing	P-value
T0	0.42	0.22	0.17	0.53	0.03	0.90	0.06	0.87
T1	N/A	N/A	0.17	0.63	0.17	0.62	0.56	0.10
T3	N/A	N/A	N/A	N/A	0.42	0.23	0.44	0.20
T6	N/A	N/A	N/A	N/A	0.19	0.57	0.42	0.23
T24	N/A	N/A	N/A	N/A	0.33	0.14	0.33	0.14

^aN/A = If in the previous time point experiments none of the blood-feeding behaviors were statistically significantly different, then the concentration was not used for the subsequent holding time experiments.

336 **Table 2.** Blood engorgement level between DEET pre-exposed or control mosquitoes.

Incubation time	DEET concentration	Cohort	Blood engorgement level ^a		P-value	
			0-3	4-5	Fisher's exact test ^b	Stepdown- Sidak ^c
T0	0.10%	Control	103	15	0.68	0.97
		Treatment	105	12		
	0.12%	Control	112	6	1	1
		Treatment	112	5		
	0.14%	Control	110	7	0.1	0.46
		Treatment	120	2		
	0.16%	Control	111	9	0.6	0.97
		Treatment	109	6		
T1	0.12%	Control	120	4	0.11	0.43
		Treatment	113	10		
	0.14%	Control	110	13	0.69	0.9
		Treatment	99	14		
	0.16%	Control	99	28	0.06	0.13
		Treatment	108	12		
T3	0.14%	Control	101	25	0.001	0.01
		Treatment	111	7		
	0.16%	Control	84	37	0.0004	0.005
		Treatment	106	14		

T6	0.14%	Control	87	30	0.002	0.02
		Treatment	110	12		
	0.16%	Control	68	54	<0.0001	0.001
		Treatment	105	13		
T24	0.14%	Control	105	15	0.03	0.24
		Treatment	113	5		
	0.16%	Control	116	7	0.07	0.38
		Treatment	119	1		

^a Engorgement grades according to the method by Pilitt and Jones.

^b As determined by two-tailed Fisher's exact test with without correction.

^c *P*-values after Stepdown-Sidak post hoc analysis/correction.

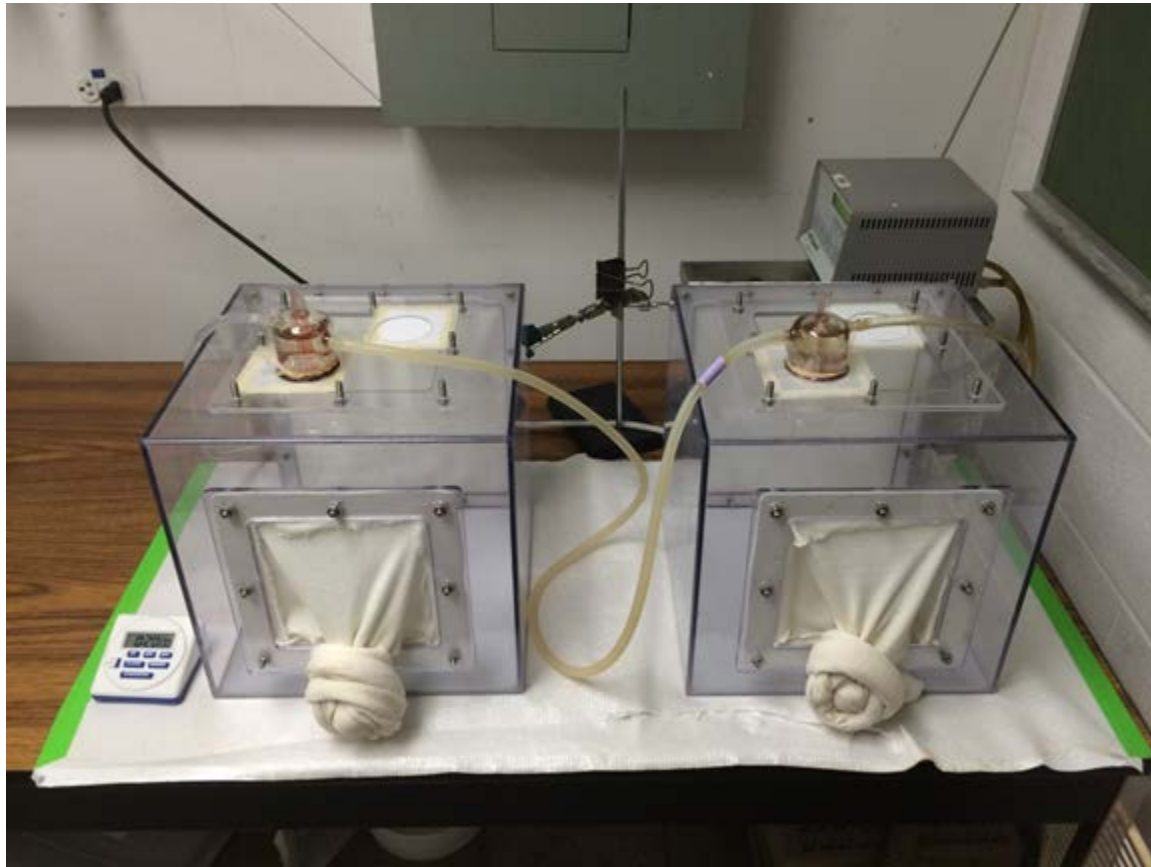
343 **Fig. 1. Plexiglas[®] box set up for blood-feeding behaviors observation.**

344 **Fig. 2. Experimental study design.**

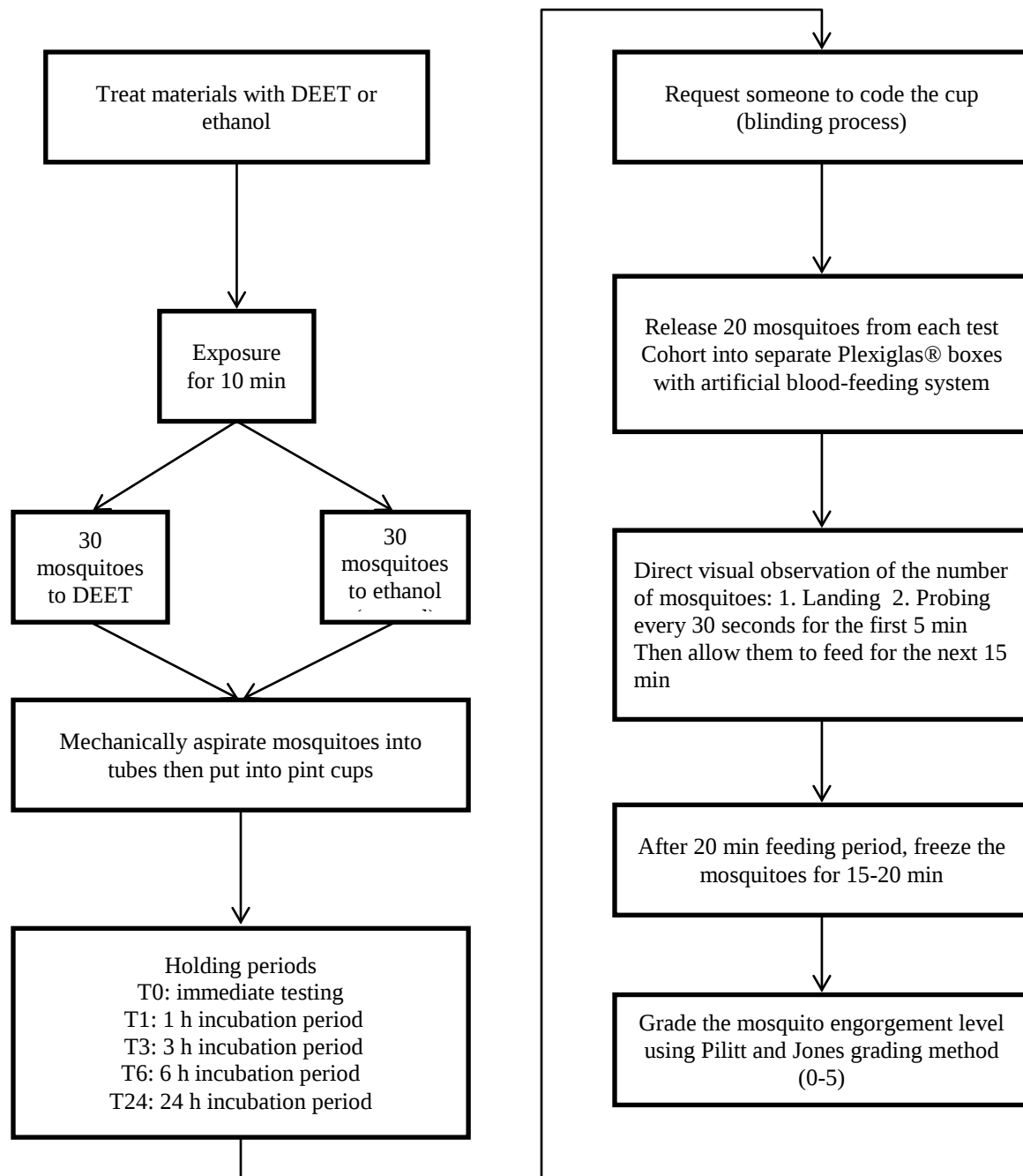
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