

Mosquito Biting Behavior: Statistical Power and Sources of Variation in Toxicity and Repellent Bioassays

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ABSTRACT Compounds thought to be effective against mosquitoes as either ‘insecticides’ or ‘repellents’ have recently been shown to contain properties of both, or possess other behavior-modifying actions. Prompted in part by these reports, we conducted posterior analyses of our data to examine some interrelated statistical issues inherent in our bioassay system. Using a modified K&D module, the responses of over 25,000 adult *Aedes aegypti* (L.) females exposed to either alphacypermethrin or DEET were compared with the responses of mosquitoes exposed to untreated controls for toxicity and biting (alphacypermethrin) or biting alone (DEET). Our analyses indicated that: (1) our bioassay system has more statistical power to determine a compound’s toxicity than its repellent qualities, (2) day-to-day variability is large and needs to be accommodated in analyses; there are other, potentially even larger sources of variability (e.g., mosquito heterogeneity) which invalidate statistical tests that are based on the assumption of binomially or multinomially distributed data (e.g., χ^2 tests), and (3) unlike biting mosquitoes exposed to DEET, the proportions of biting mosquitoes exposed to alphacypermethrin are unrelated to the proportions of concurrently tested biting controls, even after adjusting for daily variation in toxicity. Thus, there is a clear behavioral indicator in this bioassay system that the ‘repellency’ of DEET (a presumed repellent) differs in a fundamental way from that of alphacypermethrin (a presumed toxicant), which may allow the differentiation between classes of compounds based on biting behavior alone.

KEY WORDS mosquito bioassays, statistical power, variance components, biting pressure, *Aedes aegypti*

The need to protect humans from blood-sucking arthropods, particularly mosquitoes and the pathogens they transmit, has achieved renewed attention. Noteworthy among many programs are the (U.S.) President’s Malaria Initiative, for which the United States has pledged \$1.2 billion over 5 yr to combat malaria (www.fightingmalaria.gov), and the malaria control thrust of the Bill & Melinda Gates Foundation’s Global Health Program (www.gatesfoundation.org/topics/pages/malaria.aspx). Consistent with these and other programs is the need to develop new insecticides to kill mosquitoes and new repellents that offer personal protection.

Research directed at the action of insecticides and repellents has shown, in addition to a compound’s expected effects on behavior, there may be some un-

expected effects. In laboratory and field assays with three insecticides used for malaria control, Grieco et al. (2007) demonstrated that DDT also produces repellent activity, while alphacypermethrin does not repel but does act as an irritant, by causing test mosquitoes to prematurely exit laboratory and field assay arenas. Based on these studies the authors proposed a new classification scheme for chemicals used for indoor malaria control. Behavioral studies with compounds registered as repellents also indicate biological activities other than ‘repellency.’ Laboratory studies in the absence of an animal bait or blood source showed that the repellent DEET exhibits a dose-dependent mortality against a laboratory strain of *Aedes aegypti* (L.) (Licciardi et al. 2006). Other laboratory and field studies indicated that repellents have a synergistic effect when mixed with certain insecticides (Pennetier et al. 2005, 2007). In the latter study, mixtures of either DEET or KBR3023 (=picaridin) with the organophosphate insecticide pyrimiphos methyl applied to bed nets, were effective in prolonging toxicity and inhibiting blood-feeding in the Kisumu strain of *Anopheles gambiae*. Research directed at the mode of action of DEET at the cellular and sub cellular level is also of renewed interest (Ditzen et al. 2008, Pickett

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et al. 2008, Syed and Leal 2008), and the field of insect repellents has been recently reviewed (Debboun et al. 2007) as new, safer and more effective repellents are sought.

The above studies demonstrate that the interaction between mosquitoes and these bioactive compounds is complicated, and suggested to us that an analysis of some of the statistical issues common to *in vitro* bioassays might help improve our understanding of this interaction and methods used to investigate it. There are three related components to our investigation. They are (1) statistical power, the ability of a test to detect a significant effect, (2) variance decomposition, to estimate the sources of variation in mosquitoes exposed to solvent controls, and (3) assumptions underlying concurrently tested responses of mosquitoes in control versus treatment groups.

We start by noting that while few mosquitoes exposed to solvent controls die in toxicity studies, a large number of mosquitoes exposed to solvent controls do not bite in both toxicity and repellency studies. We ask how these differences in control responses affect the statistical power of these tests to determine whether a candidate compound has toxic or repellent qualities.

Controls are typically included whenever candidate compounds are tested because researchers believe day-to-day variation is substantial (Barnard and Xue 2007). Recognition of this source of variation is important, and allows researchers to test for a constant difference between the control and treatment groups (e.g., as in a paired *t*-test). We use variance decomposition methods to estimate the day-to-day variance, sampling error, and other sources of variation. Once the sources of variation are understood, better experimental designs can be developed.

An additional reason untreated controls are included whenever an experiment is conducted is the assumption that biotic and abiotic factors (see Barnard and Xue 2007) affecting biting in a repellent-exposed group will also affect controls, that is, biting rates in controls and repellents are positively correlated from experiment to experiment. However, biting can also be observed when mosquitoes are exposed to toxicants (Grieco et al. 2007), and we wondered whether this assumption (i.e., that there is a positive correlation between biting rates in controls and treatment groups from experiment to experiment) holds for all compounds affecting biting, regardless of their classification, or only if those compounds are repellents. If the latter is true, then our bioassay system can differentiate between classes of compounds based on biting behavior alone.

In this paper, we investigate these issues using analyses of toxicity and biting in an *in vitro* assay involving over 50,000 mosquitoes exposed to alphacypermethrin or an untreated (acetone) control, as well as a lesser number of mosquitoes exposed to DEET or a corresponding ethanol control. These data were collected as part of a screening program of proprietary compounds for potential mosquito biting deterrents and offer insight into bioassays of this nature.

Materials and Methods

Insects. *Ae. aegypti* (Liverpool strain) eggs were obtained from Walter Reed Army Institute of Research (Silver Spring, MD). Larvae were reared in plastic containers (50/cup) and fed daily ground Cichlid Gold fish food pellets (Kyorin Co., LTD., Himeji Japan). Adult mosquitoes were kept in plastic buckets (4.82 liters) with screened tops and maintained at $27 \pm 2^\circ\text{C}$ and $80 \pm 5\%$ RH on a 12:12 photoperiod. Adults were fed with cotton balls soaked in a 10% sucrose solution and were starved 24 h before use. The mosquitoes used in all bioassays were 5–10 d old mated, nulliparous females. Mosquitoes attracted by body heat from a hand placed near a screened opening were removed by mouth aspiration for testing; that is, mosquitoes selected for testing demonstrated an interest in feeding.

Chemicals. Alphacypermethrin, a racemate comprising (S)- α -cyano-3-phenoxybenzyl-(1R)-cis3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylate and (R)- α -cyano-3-phenoxybenzyl-(1S)-cis3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylate was obtained from BASF (Research Triangle, NC) and was checked for purity ($\geq 98\%$) using GC/MS analysis. DEET ($\geq 97\%$ N,N-Diethyl-m-toluamide) was obtained from Sigma (St. Louis, MO). Alphacypermethrin was prepared in high performance liquid chromatography (HPLC) grade acetone while DEET was prepared in ethanol.

Bioassay. The K&D module modified for *in vitro* use was used for all assays (Klun and Debboun 2000, Klun et al. 2005). The module consists of an upper unit of six chambers each containing mosquitoes that is placed upon a lower unit containing (six) corresponding reservoirs of warmed, expired human red blood cells (Walter Reed Army Medical Center, Washington, DC). Between the upper and lower units is placed a Teflon gasket, a nylon fabric treated with the chemical being bioassayed or with solvent (control), and an Edicol beef collagen membrane (Devro Inc., Sandy Run, SC) through which the mosquito is afforded an opportunity to feed. Conditions were similar to those described in Klun et al. (2005), with minor modifications that included the introduction of six mosquitoes per cell and the addition of 2.86 mg/ml of adenosine triphosphate (Sigma) to the red blood cells. Alphacypermethrin was applied to the nylon fabric at a concentration of 25 nmol/cm², while in experiments involving DEET, concentrations of 25 nmol/cm² and 50 nmol/cm² were used. Control treatments consisted of a single application (110 ml) of either acetone (in experiments using alphacypermethrin) or ethanol (in DEET experiments). Solvents in all experiments were allowed to evaporate from the nylon fabric in a chemical fume hood before the fabric was placed between the Teflon gasket and collagen membrane.

At the end of a 3 min exposure period, the number of mosquitoes biting plus those mosquitoes already engorged was recorded, and the upper part of the module was removed from the treatment (Klun et al. 2005). The number of mosquitoes knocked down (in-

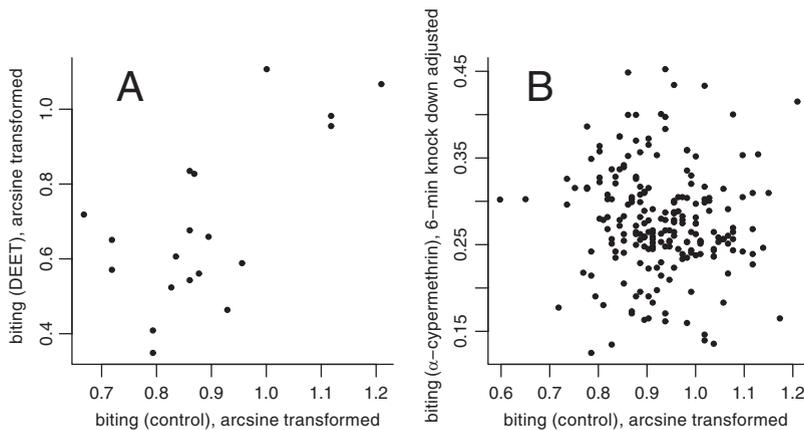


Fig. 1. Scatter plot of biting proportions of controls, and same-day and session (A) DEET exposed *Ae. aegypti*, and (B) alphacypermethrin exposed *Ae. aegypti*. Proportion of biting alphacypermethrin exposed mosquitoes has been statistically adjusted for 6 min toxicity knock down proportions.

capacitated; see White 2007) was recorded at 6 min and 1 h after this removal, and a small piece of cotton soaked with a 10% sucrose solution was placed into each cell. Mortality was recorded 24 h after the initial exposure. Each treatment was repeated 20 times, thus 120 mosquitoes were used for each exposure in a session.

Statistical Methods. A variance decomposition was conducted on a subset of the control data (mosquitoes exposed to solvent control) to estimate sources such as day-to-day variation. We systematically selected 31 d from 3 yr of data that had at least two full sessions per day (each session has data from 120 controls). Variances were calculated on arcsine transformed proportions of mosquitoes biting in each cell (i.e., $n = 6$ per cell) using the lmer function in the R statistical software system (R Development Core Team 2008). We used an arcsine transformation (Mosteller and Youtz 1961), which is better when n is small, $z = \arcsine[\sqrt{\{(y + \frac{3}{8}) / (n + \frac{3}{4})\}}]$, where y is the number of biting mosquitoes per cell and $n = 6$. While this model, where each arcsine transformed cell proportion is considered a sample from a normal distribution, provides good estimates for day-to-day and daily session-to-session variance, it does not separate sampling error from other sources of error not accounted for in the model, such as mosquito heterogeneity, cell-to-cell heterogeneity, or correlation occurring within the cell (i.e., if mosquitoes do not act independently within a cell).

The pure sampling error component can be estimated by calculating the theoretical variance of arcsine transformed binomial samples or by simulation. If this quantity is subtracted from the residual variance, the difference is the sum of the variances of all other effects not otherwise accounted for. We used simulation in the R statistical software system to calculate pure sampling error, drawing 10,000 samples with $n = 6$, and parameter $P = 0.64$ (p is the average proportion of control biting mosquitoes in this data set), and estimated this variance (pure sampling error) to be

0.038. Note that, because this is a variance stabilizing transformation, this estimate should be approximately correct for all values of p , except those close to zero or one, where the variance must approach zero.

The DEET data (at 25 nm/cm^2 and 50 nm/cm^2) were pooled after we determined that the P value for testing for the statistical difference between the two doses was very large ($P = 0.91$), that is, far from significant. These data were then used to estimate the correlation assumed to exist between mosquitoes exposed to either DEET or a solvent control on the same day.

Results

Overall, we found that the average proportion of biting mosquitoes in all solvent controls in both the alphacypermethrin and DEET experiments was only 64%, despite attempting to select hungry female mosquitoes. Of the 25,200 mosquitoes exposed to the toxicant alphacypermethrin, 81.2% were knocked down after 6 min and 98.6% were dead when examined 24-h after exposure. In an equal number of mosquitoes exposed to a solvent (acetone) control, only 1.4% were knocked down after 6 min, and 12.9% dead at the 24-h examination. Of interest is that, given the ultimately high toxicity of alphacypermethrin, a small number of mosquitoes (479; 1.9%) acquired a blood meal under our bioassay conditions.

Of the 2,124 mosquitoes exposed to DEET at either 25 nm/cm^2 or 50 nm/cm^2 , 40% bit compared with 64% biting in the solvent (ethanol) controls.

Correlation in Biting Behavior Between Solvent Controls and Either DEET or Alphacypermethrin. The estimated correlation in biting between concurrently tested mosquitoes exposed on the same day to either a solvent control or DEET was 0.67 ($P = 0.002$, $df = 17$, Fig. 1A). These results are consistent with the assumption that a measure of daily biting pressure in controls is a useful indicator of how mosquitoes will respond in a repellent treatment, and suggests that

Table 1. Components of variation in biting in cells containing six *Ae. aegypti* females exposed to a solvent control^a

Variance component	Estimate	Percent (including sampling error)	Percent (ignoring sampling error)
Day-to-day	0.0056	9.8	28.6
Session-to-session (within day)	0.0021	3.7	10.7
Other sources	0.0119	20.7	60.7
Sampling error	0.0379	65.8	-
Total	0.0576	100.0	100.0

^a Results of a variance decomposition on arcsine transformed proportions of biting mosquitoes (25,200 mosquitoes total), where the experimental unit is a six mosquito cell. Sampling error variance was estimated by simulation to be 0.0379.

using control biting pressure as a covariate will improve the sensitivity of tests to repellent differences, and is necessary if repellents are tested on different days.

This correlation in biting behavior was not found for the toxicant alphacypermethrin. Random samples of 19 sessions (to match the size of the tests with DEET), showed only about one in 10 yielded a significant correlation and in these the correlation coefficient was always estimated to be negative. To verify that this was not an artifact of the dates used to test DEET, we selected dates that matched those when DEET exposures were conducted, and found this correlation also to be not significant ($P = 0.72$, $df = 7$). As an additional check, we statistically adjusted for the toxicity of alphacypermethrin by estimating a linear model as follows. The arcsine transformed proportion of biting mosquitoes in the alphacypermethrin treatment was the dependent variable. The two independent variables were the transformed proportion of control biting mosquitoes, and the transformed proportion of mosquitoes knocked down at the 6 min observation (of the three knock down variables, this was the most strongly correlated with biting). The control biting proportion covariate was again not significant ($P = 0.30$, t -test, $df = 206$, Fig. 1B). Thus, the variability seen in biting mosquitoes in the alphacypermethrin treatment is unrelated to the proportion of control biting mosquitoes, even after statistically adjusting for the alphacypermethrin toxicity effects. This latter model had an R^2 of 0.14, so considerable unexplained variation remained after adjusting for knock down, but we know it is not related to the proportion of biting controls. Unlike the repellent DEET, the control biting pressure appears unrelated to that from the adjusted alphacypermethrin exposed mosquitoes tested during the same session.

Variance Decomposition. Results from the variance decomposition for arcsine transformed proportions of mosquitoes exposed to a solvent control are given in Table 1. The largest proportion of the error in our study is attributed to sampling (65.8%), because of our selection of the cell (=6 mosquitoes) as the experimental unit. In typical repellent and toxicity experiments, n is >100 , so sampling error is small and ignorable. There is no variance component attribut-

able to a treatment because our interest is in extrabinomial variation in controls only (i.e., no treatment mosquitoes were included).

If mosquito biting behavior truly followed a binomial distribution, sampling error should have accounted for most of the variation (i.e., approach 100%, rather than only 65.8%). However, we found that other sources of variation were large. Of the total variance (ignoring sampling error), the day-to-day variance estimate was 28.6% and session-to-session variance 10.7%. A surprisingly large 60.7% of the total variance (ignoring sampling error) was attributed to other (unknown) sources of variation. This latter category, which we cannot partition further with our data, includes mosquito heterogeneity, cell-to-cell variability (i.e., within-module variability), and module-to-module (within a session) variability. If mosquitoes do not act independently within a cell, that could also contribute to this variance component.

Discussion

Power. Since the activity of candidate repellent compounds in any bioassay system is determined by comparing biting rates of mosquitoes in treatments against biting rates in solvent controls, the statistical power of an assay (i.e., ability of a test to detect a significant effect) is diminished as the proportion of controls biting decreases for two reasons:

1. Because of the dependence of the variance of a proportion on its mean. The formula for the variance of a binomial proportion is $p(1-p)/n$, where p is the mean proportion (of biting mosquitoes) and n the number of individuals tested in the experimental unit. For fixed n , the variance is at a maximum at $P = 0.5$, and a minimum (zero) when $P = 0$ or $P = 1$. Thus, there is more power when the variance of the controls is smaller (i.e., when p for the controls is closer to one).
2. As the proportion of biting controls decreases, the potential difference between biting proportions for control and treatment mosquitoes can only decrease, because there is a hard lower limit (zero) for the proportion of treatment mosquitoes potentially biting. In comparison to using the proportion biting as a dependent variable, in toxicity tests, estimates of knockdown for controls were 1, 10, and 13% (6 min, 1 h, and 24 h, respectively). Compared against alphacypermethrin (with knock-down percentages of 82, 98, and 99% for the same times), it is evident that the toxicity tests will have far more power for the same number of tested mosquitoes.

Numeric estimates of power in a similar testing environment, also using K&D modules, along with details not discussed here, are given in Klun et al. (2008). Our results from investigating the power of tests shows that, for the same number of mosquitoes, the K&D module will have more power to determine whether a substance is toxic than whether it is repellent. This is important because any attempt to categorize a chemical by its "primary" mode of action may

not be accurate if the different modes have different statistical power. In general, for this reason a toxicological effect is likely to be detected at a lower concentration than a repellent effect. Another issue is that compounds may appear to have different modes of action depending on what organism they are tested on. For example, if the ability to detect and respond to a specific compound varies among taxa (mosquitoes versus ticks; or anopheline mosquitoes versus culicine mosquitoes), then the classification of the compound (as a repellent) will differ depending on which species is used in the bioassay.

Sources of Variation. The variance decomposition shows clearly that control mosquito biting behavior is over-dispersed relative to a binomial distribution, even when all data are taken on the same day in one session. Thus, if data collected this way are analyzed using, for instance, a two-by-two contingency table (rows are numbers biting or not biting, columns are control or treatment), the P value will be too small because it is based on the assumption that the samples are drawn from a binomial (or multinomial if there is more than one treatment) distribution, so a true null hypothesis will be rejected too often. A readable explanation for a biological audience for why this occurs is given by Kramer and Schmidhammer (1992). The largest variance component (60.7%), ignoring sampling error, was the lumping together of 'other sources' of variation we could not separate with our data. There are a large number of potential factors that could contribute to this component, such as mosquito heterogeneity, which are known to affect biting behavior and the reader is referred to Barnard and Xue (2007) and the references therein. Another potential source for this variance component is the difference between cells in a module, or between modules. Because these modules are plastic, they may become impregnated with some of the more volatile compounds used in testing. This may differ from cell to cell, and older modules may have absorbed more chemicals. However, we found no evidence that biting in controls decreased (linear regression, t -test = 1.06, $P = 0.30$, $df = 17$) over the 3 yr these data were collected, which would have been expected if toxic or repellent compounds were absorbed by the plastic.

Our variance decomposition analyses also indicated that, while day-to-day variation was large, variation from other sources was twice the day-to-day variance and demonstrates how poorly we understand the factors influencing mosquito biting. Additional research is necessary to identify the responsible factors so that these sources of variation can be reduced or recognized in the analysis. For instance, recent reports that *Drosophila* behavior is changed when grouped (Krupp et al. 2008), though unrelated to feeding behavior, suggest that feeding (or not feeding) by one mosquito may influence the feeding behavior of other mosquitoes in the same cell. If mosquito biting behavior in a cell is not independent, this would increase cell-to-cell heterogeneity. If mosquitoes do not act independently in these cells, then they also may not behave inde-

pendently in field conditions, which would affect field testing as well.

While our data are derived from the modified K&D assay system, these results should apply to other in vitro screening systems, and suggest that investigators using other assay systems need to determine the magnitude of the extra-binomial dispersion and the factors responsible. At the very least, analyses should not be conducted under the assumption that the data are binomial samples. While other in vitro bioassays are available to screen putative toxicants, the modified K&D module remains a useful tool for screening these compounds because the presence of a blood source allows one to make behavioral observations of landing, probing, and feeding that allows evaluation of the compound for behavioral effects other than insecticidal. For example, Haynes (1988) emphasizes the difference between being able to distinguish behavioral changes based upon an insect's sensory perception from their behavior following a sublethal dose of a compound; this requires direct observations.

Mosquitoes (and most other animals) avoid many naturally occurring and potentially harmful chemicals (e.g., fumes from sulfuric acid, ammonia), and have presumably developed both the ability to detect and the appropriate behaviors to avoid those irritating and toxic substances with which the species shares a history. A first step is to evolve the ability to detect the toxic substance. Thus, a synthetic "new" repellent must be sensorially similar to one of the substances mosquitoes have already evolved to avoid in nature (i.e., mosquitoes must be "preadapted" to sense and to avoid the new compound for it to act as a repellent). Arguments similar to this have been advanced in the discussion of new host acquisition by phytophagous insects, albeit from the standpoint of attractants (Ben-venbaum 1990).

However, this would not necessarily be true for new toxicants, which mosquitoes may or may not detect. In the short run, a new toxicant without repellent properties would be effective for killing mosquitoes; however, given sufficient time one would expect mosquitoes to evolve either sensitivity to the compound, so they can avoid it, or physiological (metabolic) resistance, so the compound would no longer be a toxicant. While we know of no relevant mosquito studies, the evolution of chemical warfare between phytophagous insects and their plant hosts is well documented (Schoonhoven et al. 2006).

"Repellents" can have different modes of action; feeding and other behaviors may be altered in different ways depending on the primary mode of action of the chemical under investigation. This is important because of the assumptions under which behavioral tests for these compounds are conducted. Because any large day-to-day variation in biting pressure can make subsequent analyses difficult, a common assumption made is that biting rates in controls and repellents are positively correlated, that is, on days when most controls bite, there are also more mosquitoes biting in the repellent treatments than on days when fewer controls bite. While we found this relationship to hold for

DEET, it did not for alphacypermethrin. The percent of mosquitoes biting in the alphacypermethrin treatment was unrelated to the biting pressure of that day, even after adjusting for daily differences in mortality in the alphacypermethrin treatment. This unexpected outcome demonstrates that the modes of action of different compounds affect behavioral responses and suggests that factors influencing normal biting pressure (measured in controls) become somehow decoupled in mosquitoes exposed to alphacypermethrin. It would be interesting to determine if these relationships hold true for other compounds classified as 'repellents' or 'toxicants' because it may allow for a distinction to be made on new compounds based on biting behavior alone.

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References Cited

- Barnard, D., and R. d. Xue. 2007. Biometrics and behavior in mosquito repellent assays, pp. 111–124. *In* M. Debboun, S. P. Frances SP, and D. Strickman [eds.], *Insect Repellents: Principles, Methods, and Uses*. CRC, Baton Rouge, LA.
- Berenbaum, M. R. 1990. Evolution of specialization in insect Umbellifer associations. *Annu. Rev. Entomol.* 35: 319–343.
- Debboun, M., S. P. Frances, and D. Strickman. 2007. *Insect repellents: principles, methods, and uses*. CRC, Baton Rouge, LA.
- Ditzen, M., M. Pellegrino, and L. B. Voshall. 2008. Insect odorant receptors are molecular targets of the insect repellent DEET. *Science* 319: 1838–1842.
- Grieco, J. P., N. L. Achee, T. Chareonviriyaphap, W. Suwonkerd, K. Chauhan, M. Sardelis, and D. R. Roberts. 2007. A new classification system for the Action of IRS chemicals traditionally used for malaria control. *PLoS ONE* 2: e716 (doi: 10.1371/journal.pone.0000716).
- Haynes, K. 1988. Sublethal effects of neurotoxic insecticides on insect behavior. *Annu. Rev. Entomol.* 33: 149–168.
- Klun, J. A., and M. Debboun. 2000. A new module for quantitative evaluation of repellent efficacy using human subjects. *J. Med. Entomol.* 37: 177–181.
- Klun, J. A., M. Kramer, and M. Debboun. 2005. A new in vitro bioassay system for discovery of novel human-use mosquito repellents. *J. Am. Mosq. Contr. Assoc.* 21: 64–70.
- Klun, J. A., M. Kramer, A. Zhang, S. Wang, and M. Debboun. 2008. A quantitative in vitro assay for chemical mosquito deterrent activity without human blood cells. *J. Am. Mosq. Contr. Assoc.* 24: 508–512.
- Kramer, M., and J. Schmidhammer. 1992. The chi-squared statistic in ethology: use and misuse. *Anim. Behav.* 44: 833–841.
- Krupp, J. J., C. Kent, J. C. Billeter, R. Azanchi, A.K.C. So, J. A. Schonfeld, B. P. Smith, C. Lucas, and J. D. Levine. 2008. Social experience modifies pheromone expression and mating behavior in male *Drosophila melanogaster*. *Curr. Biol.* (doi: 10.1016/j.cub.2008.07.089).
- Licciardi, S., J. P. Herve, F. Darriet, J. M. Hougard, and V. Corbel. 2006. Lethal and behavioral effects of three synthetic repellents (DEET, IR3535 and KBR 3023) on *Aedes aegypti* mosquitoes in laboratory assay. *Med. Vet. Entomol.* 20: 288–293.
- Mosteller, F., and C. Youtz. 1961. Tables of the Freeman-Tukey transformations for the binomial and Poisson distributions. *Biometrika* 48: 433–440.
- Pennetier, C., V. Corbel, and J.-M. Hougard. 2005. Combination of a non-pyrethroid insecticide and a repellent: a new approach for controlling knockdown-resistant mosquitoes. *Am. J. Trop. Med. Hyg.* 72: 739–744.
- Pennetier, C., V. Corbel, P. Boko, A. Odjo, R. N'Guessan, B. Lapied, and J. M. Hougard. 2007. Synergy between repellents and non-pyrethroid insecticides strongly extends the efficacy of treated nets against *Anopheles gambiae*. *Malaria J.* 6: 38 (doi: 10.1186/1475-2875-6-38).
- Pickett, J., M. A. Birkett, and J. C. Logan. 2008. DEET repels ORNery mosquitoes. *Proc. Natl. Acad. Sci. U.S.A.* 105: 13195–13196.
- R Development Core Team. 2008. The R manuals. (<http://cran.r-project.org/manuals.html>).
- Schoonhoven, L. M., J.J.A. van Loon, and M. Dicke. 2006. *Insect-plant biology*, 2nd ed. Oxford University Press, Oxford, United Kingdom.
- Syed, Z., and W. S. Leal. 2008. Mosquitoes smell and avoid the insect repellent DEET. *Proc. Natl. Acad. Sci. U.S.A.* 105: 13598–13603.
- White, G. B. 2007. Terminology in insect repellents, pp. 31–46. *In* M. Debboun, S. P. Frances, and D. Strickman [eds.], *Insect Repellents: Principles, Methods, and Uses*. CRC, Baton Rouge, LA.

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