Journal of Medical Entomology, 54(3), 2017, 631–637
doi: 10.1093/jme/tjw207
Advance Access Publication Date: 27 December 2016
Research article

Comparative Evaluation of a Silicone Membrane as an Alternative to Skin for Testing Mosquito Repellents

Natasha M. Agramonte,[1,2,3] Salvador A. Gezan,[4] and Ulrich R. Bernier[2]

[1]Department of Entomology & Nematology, Emerging Pathogens Institute, University of Florida, Gainesville, FL 32608 (nme@epi.ufl.edu), [2]U.S. Department of Agriculture-Agricultural Research Service, Center for Medical, Agricultural, and Veterinary Entomology, Gainesville, FL 32608 (uli.bernier@ars.usda.gov), [3]Corresponding author, e-mail: nme@epi.ufl.edu, and [4]School of Forest Resources and Conservation, Institute of Food and Agricultural Sciences, University of Florida, Gainesville, FL 32611 (sgezan@ufl.edu)

Subject Editor: Lyric Bartholomay

Received 17 August 2016; Editorial decision 1 November 2016

Abstract

Repellents prevent mosquito bites and help reduce mosquito-borne disease, a global public health issue. Laboratory-based repellent bioassays predict the ability of compounds to deter mosquito feeding, but the variety of repellent bioassays and statistical analysis methods makes it difficult to compare results across methodologies. The most realistic data are collected when repellents are applied on the skin; however, this method exposes volunteers to chemicals and mosquito bites. Silicone membranes were investigated as an alternative to human skin in assays of repellent efficacy. Results from module system bioassays conducted in vitro with a silicone membrane were compared with in vivo bioassays conducted with *N,N*-diethyl-3-methylbenzamide (referred to as DEET), 1-piperidinecarboxylic acid 2-(2-hydroxyethyl)-1-methylpropylester (referred to as Picaridin), ethyl 3-[acetyl(butyl)amino]propanoate (referred to as IR3535), and *para*-menthane-3,8-diol (referred to as PMD) applied directly on the skin of the leg. No significant difference in mosquito feeding was found when comparing skin and volunteer-worn membrane controls using blood; however, feeding was significantly lower in unworn membrane controls using either 10% sucrose or blood, indicating that worn membranes are a possible surrogate for untreated human skin. Pooled data from six volunteers were used to generate dose–response curves of blood-feeding activity. Results from skin-applied repellents were modeled to determine if membranes could provide a predictive correlate for skin. Goodness-of-fit comparisons indicated that the nonlinear dose–response curves for the skin and membrane differed significantly for DEET and Picaridin, but did not differ significantly for IR3535 and PMD. With knowledge of the dose–response relationships and further modifications to this system, the membrane-based tests could be used for standardized repellent testing with infected vectors.

Mosquito-borne diseases are a major public health problem, and *Aedes aegypti* (L.) is a global vector of dengue, Zika, chikungunya, and yellow fever, all of which cause severe human morbidity and mortality (Gratz 1999, Roth et al. 2014). Insect repellents applied to skin are used as a means of personal protection to prevent mosquito bites, and thus, reduce disease transmission caused by arthropod vectors (Moore and Debboun 2007). In vitro methods for evaluation of mosquito repellents provide a fast, safe, and inexpensive way to test chemicals regardless of whether toxicological analysis has been conducted to determine whether it is permissible for evaluation of the chemical directly on skin. There are limitations that should be understood when results from these methods are compared with each other or even more importantly when results are used to estimate potential performance of a repellent in the field. It is not known how well results from in vitro studies correlate with those obtained from in vivo studies. Because by its nature a treatment is not directly tested on the target host in an in vitro test, out of convenience or necessity, extrapolation of these results to in vivo systems without an understanding of the degree of correlation limits their usefulness. Few studies have been conducted to test how comparable the results are from in vitro and in vivo methods or how accurate an estimation in vitro methods provide of repellent performance on skin.

In vivo methods also have disadvantages that complicate their use. These studies require human volunteers willing to subject themselves to an accumulation of mosquito bites and the potential for allergic reactions. It is also difficult to perform experiments with a high degree of statistical power because of the need for large sample sizes, particularly when repellent efficacy is measured by the duration or quantity of a compound that prevents mosquitoes from biting through the skin. Although testing in the field is the best predictor of how candidate

repellents will perform when used outdoors, the risk of exposure to pathogens carried by wild mosquitoes makes it impractical and unethical to perform these experiments in some geographical locations.

Despite the drawbacks of in vitro methods and their unpopularity with some researchers, they remain valuable tools for mosquito repellent research because they can be used to predict or screen for repellency before in vivo testing, or when in vivo testing is not feasible or very difficult to conduct. The desire to produce an accurate rapid-screening method has produced a myriad of in vitro testing methods in comparison to the handful of in vivo methods (Butler et al. 1984, Cockcroft et al. 1998, Dogan and Rossignol 1999, Klun and Debboun 2000, Weldon et al. 2003, Rutledge and Gupta 2004, Bernier et al. 2005, Klun et al. 2005, Barnard et al. 2007, Logan et al. 2010). Modification of some of the most promising in vitro methods may result in a test procedure by which in vivo test results could be reliably predicted without the need for human volunteers as test subjects. With the ability to identify the best potential repellents through screening, the cost for conducting toxicological analysis of a large set of novel repellent compounds could also be minimized. Furthermore, the United States Environmental Protection Agency Human Studies Review Board (HSRB) has expressed concerns over the use of humans in repellent studies, and the outcomes of this study could be of interest to the United States Environmental Protection Agency (EPA) for the development of future rules on the registration of repellents (EPA 2000). The objective of this study was to compare the dose–response curves of four common mosquito repellent-active ingredients tested in vivo on human skin and in vitro on a silicone membrane to determine if the membrane system is a good surrogate for skin with respect to the evaluation of repellent efficacy.

Materials and Methods

Mosquito Rearing and Selection

Mosquitoes used in all bioassays were female *Ae. aegypti* (Orlando strain, 1952) from the colony maintained at the Center for Medical, Agricultural, and Veterinary Entomology (CMAVE), a center within the United States Department of Agriculture, Agricultural Research Service (USDA-ARS) in Gainesville, FL. Pupae were obtained from the onsite colony and maintained in laboratory cages until ready for use in experiments. Newly emerged mosquitoes were maintained ad libitum on a 10% sucrose solution at 25–28°C ambient temperature, 60–80% RH, and a photoperiod of 14:10 (L:D) h. Nulliparous female mosquitoes aged 6–11 d were preselected for host-seeking behavior from stock cages using a hand-draw box and a collection trap (Posey and Schreck 1981). Female mosquitoes from the collection trap were then transferred to a smaller cage (30.48 by 30.48 by 30.48 cm^3) from which the mosquitoes were sorted into groups of 10 by mechanical aspiration into acrylic holding tubes (15 cm in length, 1.25 cm in diameter). Each tube contained ~10 mosquitoes and was sealed by a screen gauze on one end and by a small cork (Size 1, Fisher Scientific, Catalog No. 07781D, Hampton, NH) at the other end. Mosquitoes in the tubes were allowed to acclimate for 15–20 min (Barnard et al. 2007) and then blown via exhalation into an empty module chamber before being used for testing.

Chemical Treatments and Control

Repellent treatments were all technical grade and included *N,N*-diethyl-3-methylbenzamide, 97% purity (Aldrich, CAS#134-62-3), hereafter referred to as "DEET"; ethyl 3-[acetyl(butyl)amino]propanoate, 98% purity (Merck, CAS#52304-36-6), hereafter referred to as "IR3535"; 1-piperidinecarboxylic acid 2-(2-hydroxyethyl)-1-methylpropylester, 98% purity (Saltigo, CAS#119515-38-7), hereafter referred to as "Picaridin"; and *para*-menthane-3,8-diol, 98% purity (Bedoukian, CAS#42822-86-6) hereafter referred to as "PMD." Test solutions were made from serial dilutions of these four chemicals by adding 1 ml of denatured ethanol (Acros, CAS#64-17-5) into 1 ml of the previous concentration of chemical to produce a range of concentrations (7,840, 3,920, 1,960, 980, 490, 245, 123, 61, 31, 15, and 8 nmol/cm^2) when applied to a 14.19-cm^2 treatment area. The control treatment consisted only of denatured ethanol.

Alterations Made to Module From Previous Designs

Several modifications were made to the module and protocols described previously by Klun and Debboun (2000) and Weldon et al. (2003). Modifications made to the original Klun and Debboun module by Weldon et al. were an increase in the internal volume of each chamber from 100 to 125 cm^3, an increase in the spacing between the chambers from 0.25 to 1.25 cm, and a reduction in the circular aperture to a diameter of 4.25 cm from a 3 by 4 cm rectangular aperture (2003). Modifications made to the Weldon module system for this study included an increase in the number of mosquitoes placed in each chamber from 5 to 10; this increase allowed for more precise feeding proportions to be recorded at each concentration level. A glass plate with drilled holes in line with the blood wells and apertures of the feeding module was added. The glass plate was positioned under the feeding module to act as an inert barrier between the feeding module and the loading module or between the feeding module and the skin of the volunteers (Fig. 1a and b). This glass plate also alleviated concerns of module contamination by preventing the absorption of chemicals into the PLEXIGLAS bottom of the module from which contact with the chemicals was most likely to occur. Elastic rubber bands were added to the sliding doors to prevent accidental opening of the chamber doors by the volunteers during testing.

A procedural modification in this study was the use of layering of repellent treatments of increasing dose on the skin or silicone membrane. This procedure allowed six replicates of a candidate repellent concentration to be tested concurrently. In previous studies, different doses were evaluated in each chamber. By testing the same concentration across all chambers, concerns over interactive effects from different concentrations in adjacent chambers are minimized.

To ensure that the tests were run for the same duration in all chambers, carbon dioxide (CO_2) was pumped into the chambers using a multiport connector fashioned from nylon tubing and nylon barbed Tee connectors arranged in parallel. This allowed for CO_2 to be simultaneously pumped into multiple chambers for the purpose of anesthetizing the mosquitoes at the conclusion of each testing session. Using this apparatus, three or six chambers could be tested concurrently, reducing testing time at least threefold compared with the previous testing scheme.

In Vivo Module Bioassays on Skin

For these bioassays, the proportion of *Ae. aegypti* mosquitoes blood-feeding on the thighs of six human volunteers (four male, two female) was determined for a range of concentrations of the repellent chemicals DEET, IR3535, Picaridin, and PMD. No specific pretest washing procedures were requested of the volunteers. Ten nulliparous female *Ae. aegypti* mosquitoes (6–11 d) were loaded into chambers ($5 \times 5 \times 5$ cm^3) in the previously described loading module (Klun and Debboun 2000, Weldon et al. 2003, Rutledge and Gupta 2004, Klun et al. 2005). Each chamber in the loading

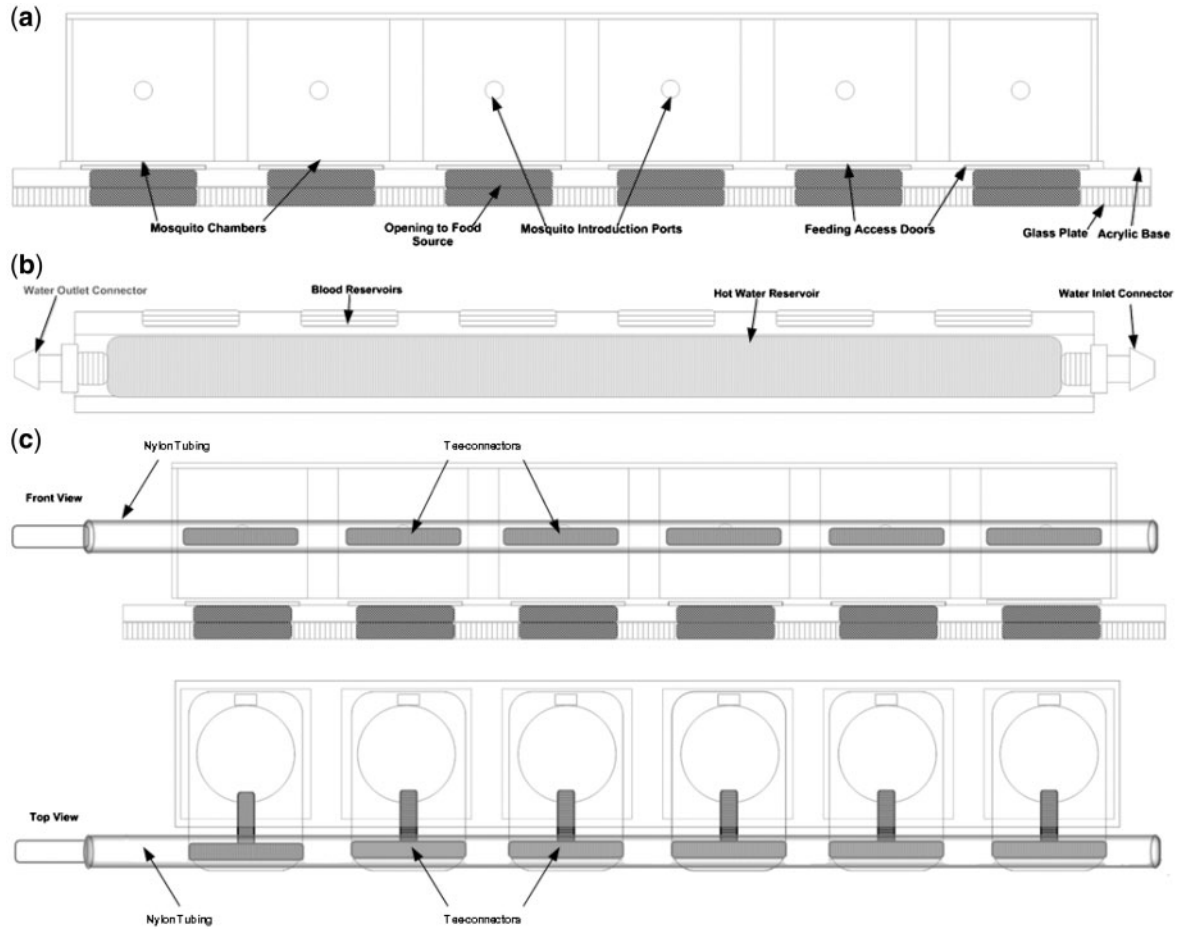

Fig. 1. (**a**) Schematic of mosquito loading module with six testing chambers. (**b**) Schematic of mosquito feeding module with 6-well receptacles for blood feeding. (**c**) Front and side views of CO^2 tubing connected in parallel with the front of the six testing chambers of the mosquito loading module.

module had two apertures, a 4.25-cm-diameter circular aperture at the bottom of each chamber, covered by a sliding door, and a small 1.5-cm-diameter circular aperture on the front of each chamber that was sealed by a small cork.

The areas on the thighs where the test solution was to be delivered were demarcated with 4.25-cm-diameter circles corresponding to the six chambers. A 50-µl ethanol-diluted dose of DEET, IR3535, Picaridin, or PMD was applied to the six circles in successive layered doses from the lowest to highest concentration, i.e., 8 onto 8 nmol/cm^2 to achieve 15 nmol/cm^2 concentration, which has been rounded for clarity. The first treatment applied in any set of tests was ethanol control, which was used to establish the baseline for mosquito feeding behavior. Each application was allowed to dry for 3–5 min to allow the ethanol solvent to evaporate. The loading module was then lined up with the demarcated areas, with a glass spacer inserted between the module and the skin to prevent direct contact of the module with the chemicals. A sliding door was opened under one-half of the chambers (three) at a time to expose mosquitoes in those chambers to the repellent for a 3-min period. At the end of the exposure period, the mosquitoes were knocked down with CO_2 via insertion of the nylon tubing into the corked holes, removed from the chamber with an aspirator, and crushed to record the proportion of mosquitoes that blood-fed. This procedure was repeated with the other three chambers and with all concentrations of the repellent until a concentration was applied that resulted in no feeding by the mosquitoes. Volunteers in all repellent tests provided written informed consent for participation, and the study was approved by UF IRB-01 (Project # 636-2005).

In Vitro Module Bioassays Using Silicone Membranes Treated With Skin Odors

Each well in the feeding module was filled with 7 ml of citrated bovine blood, maintained at ~37°C by a continuous flow of hot water through the feeding chamber with a circulating water bath. Before testing, the volunteers wore silicone membrane strips against the upper thigh for 3–4 h, which were held in place with an Ace elastic bandage to promote sweating and transfer of skin chemicals. The silicone membranes were handmade and generously donated by Paul Weldon, a colleague from the Smithsonian Institute, for the purposes of this research project. The membranes were made by spreading silicone over a nylon mesh fabric and sandwiched between two pieces of laboratory stretch film before being hand-cranked through a mechanical press to a thickness of 0.1 mm, according to the method of Butler et al. (1984).

The volunteer-worn silicone membranes were then placed across the six wells of the feeding module and were in contact with the blood. The glass spacer was placed over the silicone membranes, leaving only the membrane-covered well area exposed. A 50-µl ethanol-diluted dose of DEET, IR3535, Picaridin, or PMD was applied to the six circles on the silicone membrane above each well. Chemical doses were layered on top of previous applied doses to

Table 1. Repellency ED_{50} estimates (95% CI) (nmol/cm^2), hill slope, and R^2 calculated from the nonlinear regression fit of sigmoidal variable-slope dose–response curves for skin and silicone membranes, pooled from six subjects

Chemical	Skin ED_{50} (nmol/cm^2)	Hill slope	R^2 (df)	Membrane ED_{50} (nmol/cm^2)	Hill slope	R^2 (df)
DEET	98.7 (55.8–174.7)	2.91	0.40 (34)	430.2 (281.7–657.0)	1.23	0.75 (56)
Picaridin	109.9 (36.2–333.5)	1.17	0.43 (46)	476.7 (287.9–789.4)	1.21	0.66 (56)
IR3535	114.0 (72.7–178.9)	1.97	0.66 (42)	353.3 (136.7–912.6)	0.78	0.61 (54)
PMD	110.7 (66.6–183.9)	2.34	0.52 (38)	413.0 (164.9–1035)	0.98	0.46 (52)

achieve the range of concentrations used in the six demarcated areas. The first treatment applied in any set of tests was the ethanol control to establish the baseline for mosquito feeding behavior. Each new application of a concentration was allowed to dry for 3–5 min to allow for the ethanol to evaporate. The loading module was placed onto the feeding module and lined up to cover each well. A sliding door was opened under all six chambers to expose the mosquitoes to the repellent treatment for a 3-min period. At the end of the exposure period, the mosquitoes were knocked down with CO_2 via the corked hole, removed from the chamber with an aspirator, and crushed to record the proportion of mosquitoes that blood-fed. This process was repeated with increasing concentrations of the repellent until a concentration was applied that resulted in no feeding by the mosquitoes.

Statistical Analysis. Data from the six volunteers were pooled for each of the four repellent chemicals and at each of the concentration levels to reduce effects that were the result of biological testing and to minimize person-to-person variability in attraction of the mosquitoes. The pooled data from the repellency bioassays were analyzed by fitting a generalized linear mixed model using a binomial distribution with a probit link. The following is the equation for the fitted model:

$$\begin{aligned}\text{probit}(\text{y/n}) \;=\; & \mu + Chem + Medium + Chem \times Medium \\ & + \log Dose + Chem \times \log Dose + \\ & Medium \times \log Dose + Chem \times Medium \times \log Dose + e\end{aligned}$$

where μ is the overall mean, *Chem* is the fixed effect of the chemical (DEET, Picaridin, IR3535, and PMD), *Medium* is the fixed effect of the medium (skin and silicone membrane), *Chem* × *Medium* is the interaction between chemical and medium, *logDose* is a covariate corresponding to log-transformed dose expressed as $log(Dose + 100)$, and the other terms correspond to interactions with this variant. The random error e was defined as $e \sim N(0, \sigma^2)$. In addition, an overdispersion parameter was considered for this model. The above model was fitted using the procedure GLIMMIX as implemented in SAS 9.2 (SAS 2012). The significance of the model term effects was evaluated using an approximated F test with a significance level of $\alpha = 0.05$. Nonlinear regression analysis was performed in GraphPad Prism 6.02 software (Prism) using sigmoidal, four-parameter, dose–response fit with a variable slope to compare the dose–response curves for each group (skin vs silicone membrane; GraphPad Software 2013). The ED_{50} estimates with 95% confidence limits were generated from Prism. A Mann–Whitney U test was used to compare the untreated skin with untreated silicone membrane blood-feeding data for differences among means.

Results

The results from the F tests for the fitted model indicate that there were significant differences for all effects accounted for in the fitted model. The effects considered were *Chem*, which was the effect of the effect of the chemical treatments (DEET, Picaridin, IR3535, and PMD); *Medium*, which was the effect of the medium (skin or silicone membrane); and *logDose*, which was the effect of the log-transformed dose. Significant effects for *Chem* (F (3,71) = 6.48, $P = 0.0006$), *Medium* (F (1,71) = 83.23, $P < 0.0001$), and *logDose* (F (1,71) = 646.71, $P < 0.0001$) were found. In addition, all interactions were significant for model terms *Chem* × *Medium* (F (3,71) = 5.30, $P = 0.0023$), *logDose* × *Chem* (F (3,71) = 7.05, $P = 0.0003$), *logDose* × *Medium* (F (1,71) = 101.93, $P < 0.0001$), and the three-way interaction *logDose* × *Chem* × *Medium* (F (3,71) = 5.91, $P = 0.0012$).

This indicates that the additive model is not useful as a predictive tool for determining repellent activity on the skin from data collected on the silicone membrane.

Comparisons between skin and the silicone membrane were also made using ED_{50} estimates, calculated from the nonlinear regression fit of sigmoidal variable-slope dose–response curves for DEET, Picaridin, IR3535, and PMD (Table 1). An F test comparing the goodness of fit for the $logED_{50}$ values indicated that the nonlinear dose–response curves for the skin were significantly lower than those for the membrane with both DEET, where F (1,90) = 4.97, $P = 0.028$, and Picaridin, where F (1,96) = 5.04, $P = 0.027$. An F test comparing the goodness of fit for the $logED_{50}$ values indicated that the nonlinear dose–response curves for the skin and membrane did not differ significantly from each other with IR3535, where F (1,102) = 1.80, $P = 0.182$, and with PMD, where F (1,90) = 2.03, $P = 0.158$. Based on the ED_{50} estimates, the ranked order for the repellents applied to skin from most efficacious to least was DEET > Picaridin > PMD > IR3535, although based on their overlapping 95% confidence intervals, these treatments do not significantly differ from each other at this dose. Based on the ED_{50} estimates, the ranked order for the repellents applied to membrane from most efficacious to least was IR3535 > PMD > DEET > Picaridin, although based on their overlapping 95% confidence intervals, these treatments do not significantly differ from each other at this dose. The ED_{50} values for DEET on skin (98.7) and membrane (430.2) differed by a ratio of 4.4 (Table 1, Fig. 3). The ED_{50} values for Picaridin on skin (109.9) and membrane (476.7) differed by a ratio of 4.3 (Table 1, Fig. 4). The ED_{50} values for IR3535 on skin (114.0) and membrane (353.3) differed by a ratio of 3.1 (Table 1, Fig. 5). The ED_{50} values for PMD on skin (110.7) and membrane (413.0) differed by a ratio of 3.7 (Table 1, Fig. 6).

A comparison of baseline attraction using the Mann–Whitney U test revealed that the mean blood-feeding percentage for the control was 59.16% (SEM ± 5.25%) for in vivo and 56.17% (SEM ± 3.84%) for in vitro, which was not a statistically significant difference (U = 244.5, $P = 0.376$) (Fig. 2). Preliminary tests conducted with unworn silicone membranes using either blood or a 10% sucrose solution treated with red food coloring failed to adequately attract mosquitoes, i.e., levels of only 40.3 or 12.8% blood-feeding, respectively in the module. The Mann–Whitney U test

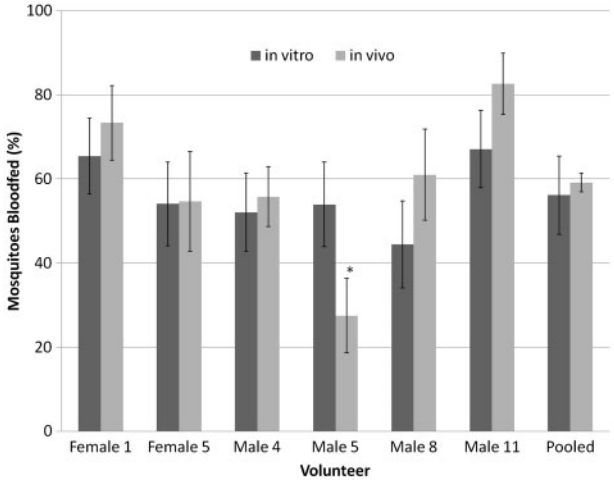

Fig. 2. Baseline attraction (% blood-fed on control) for the volunteers, both individually (Female 1, Female 5, Male 4, Male 5, Male 8, Male 11) and pooled, comparing in vivo versus in vitro mosquito feeding. Asterisk indicates individual where controls differed from each other significantly ($P \leq 0.05$). Error bars indicate SEM.

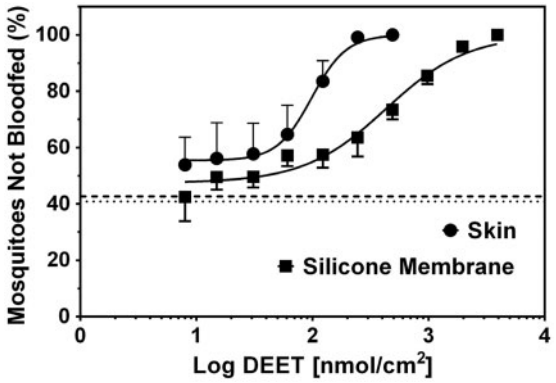

Fig. 3. Nonlinear dose–response curves indicating the percentage of mosquitoes repelled, i.e., not blood-fed, by DEET on skin (in vivo) and on silicone membrane (in vitro). Error bars indicate SEM. Dotted line indicates percent blood-feeding for skin control. Dashed line indicates percent blood-feeding for silicone membrane control.

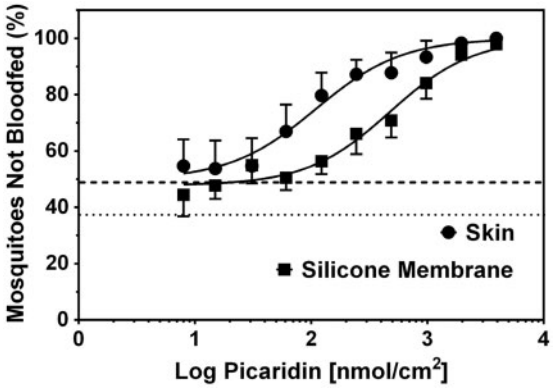

Fig. 4. Nonlinear dose–response curves indicating the percentage of mosquitoes repelled, i.e., not blood-fed, by Picaridin on skin (in vivo) and on silicone membrane (in vitro). Error bars indicate SEM. Dotted line indicates percent blood-feeding for skin control. Dashed line indicates percent blood-feeding for silicone membrane control.

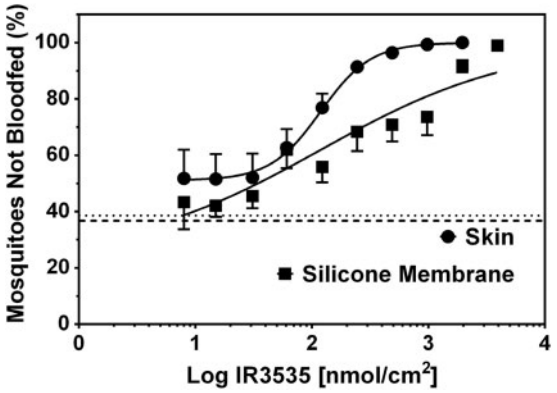

Fig. 5. Nonlinear dose–response curves indicating the percentage of mosquitoes repelled, i.e., not blood-fed, by IR3535 on skin (in vivo) and on silicone membrane (in vitro). Error bars indicate SEM. Dotted line indicates percent blood-feeding for skin control. Dashed line indicates percent blood-feeding for silicone membrane control.

indicated that there were significant differences when comparing the skin and the unworn silicone membrane with either 10% sucrose ($U = 9$, $P = 0.0003$) or blood ($U = 136.5$, $P = 0.043$), but did not indicate differences when comparing the skin and the volunteer-worn membranes ($P > 0.05$). When examined individually, one volunteer (Male 5) had a significantly lower number of mosquitoes blood-feeding on skin versus silicone membrane without repellent (Fig. 2), using a paired, two-sample *t*-test for means where the two-tailed $P = 0.016$ ($df = 3$), and because of the high variability in individual data, only the pooled data were used for further analysis.

Discussion

Two surface media, human skin, and silicone membranes that had been worn by volunteers on their skin, were used to evaluate the blood-feeding behavior of female mosquitoes exposed to four repellent chemicals in a laboratory setting. Because the baseline attraction levels were not statistically different for these surfaces, the blood-feeding behavior of the mosquitoes on the silicone membrane supports the hypothesis that a membrane system, where membranes are worn by volunteers, may be a surrogate for testing on humans in the laboratory. Volunteers wore the silicone membranes for 3–4 h before testing to transfer some of the attractive skin chemicals onto the silicone membranes. The blood-feeding behavior of the unworn silicone membrane tested using both blood (40.3%) and a 10% sugar solution (12.8%) differed significantly from that of control treatments on human skin (59.16%). However, the blood-feeding behavior of the pre-worn silicone membranes (56.17%) did not differ significantly from that of control treatments on human skin. Cockcroft et al. (1998) found similarities in attraction when the probing behavior of *Ae. aegypti* using the arm-in-cage method was compared with that using an unworn collagen membrane; however, this was not the case in our study. Cockcroft et al. examined the probing behavior, which is more difficult to accurately measure, as multiple probing events can occur by the same mosquito and probing behavior is not always indicative of a successful feeding event. Also, control data were not provided to support the similarity of untreated skin compared with untreated collagen membrane; the

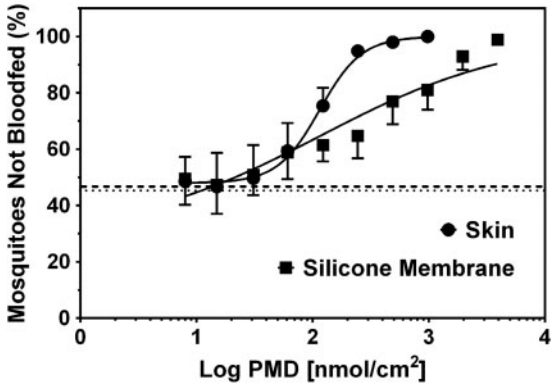

Fig. 6. Nonlinear dose–response curves indicating the percentage of mosquitoes repelled, i.e., not blood-fed, by PMD on skin (in vivo) and on silicone membrane (in vitro). Error bars indicate SEM. Dotted line indicates percent blood-feeding for skin control. Dashed line indicates percent blood-feeding for silicone membrane control.

assumption was made owing to similar ED_{50} estimates for DEET. Without knowledge of the shape of the dose–response curve, it is difficult to determine whether his untreated collagen membranes were ideal analogs for human skin.

All four repellents required on average of four times higher concentrations on the silicone membrane to reach 50% protection from mosquito blood-feeding, i.e., ED_{50}. An explanation for this difference might be the loss of repellent chemical from the surface of the silicone membrane into the blood via migration through the membrane and dissolution into the blood. This is interesting because it is unclear whether the silicone membrane became more desirable to the mosquitoes than the skin at these doses or if perhaps the skin became less desirable to the mosquitoes. Natural repellents and attraction-inhibitors have been documented in the exudate of humans (Bernier et al. 2002, 2005, 2007; Logan et al. 2008). In addition, these natural allomones have been found to vary among individuals (Ellin et al. 1974; Sastry et al. 1980; Schreck et al. 1990; Bernier et al. 1999, 2000, 2002, 2007). Perhaps the mixture of these naturally repellent or inhibiting chemicals produced by the skin, when mixed with the higher doses of repellents applied during the laboratory test, reduced the total amount of repellent needed to prevent blood-feeding by the female mosquitoes. Although the silicone membranes worn on the skin of the volunteers appear to have successfully transferred skin chemicals onto the silicone membranes owing to the increased blood-feeding by the mosquitoes on the worn membranes, it is unknown whether any of these allomonal compounds were transferred or if these are produced at higher rates when humans undergo stress, i.e., being bitten by mosquitoes, similar to a plant's use of defensive chemicals for feeding deterrence by insects (Karban and Myers 1989).

The interaction of human skin-chemicals with the repellent compounds was not explicitly explored in this study, but it could be further examined in additional studies with the silicone membrane module system. By applying a standardized human-derived blend of chemicals to the silicone membrane along with repellent treatments, the silicone membrane system could be examined to see if the chemically treated membrane produces results similar to the skin at dose applications >31 nmol/cm^2.

Preliminary, unpooled data from some of the individual volunteers (unpublished) showed evidence of an increase in feeding behavior, which was inconsistent with the expected response to increased surface concentrations of repellent chemical. This occurred at a concentration level below the ED_{50} estimates for the skin and silicone membrane curves. This effect was minimized by the pooling of the data from all volunteers. This unexpected result may be because of the attractive properties of repellent chemicals at very low doses, which has previously been documented with DEET (Mehr et al. 1990, Dogan and Rossignol 1999, Bernier et al. 2005). Logan et al. also observed the attraction of *Ae. aegypti* to low-dose repellents for octanol, nonanal, decanal, and 6-methyl-5-hepten-2-one (2010). The absorptive properties of the silicone membrane were not investigated in this study, but it is possible that attractive or non-attractive human odorant chemicals bound to the silicone membrane in different ratios than they are typically found on the skin. Further studies with lower doses of Picaridin, IR3535, and PMD should be conducted to test whether this phenomenon is occurring with these compounds as well.

Several of the following benefits can be derived from using an in vitro testing method in the laboratory instead of performing testing directly on human volunteers: lower risk to humans by not subjecting them to mosquito bites, lower exposure to compounds of unknown toxicity, ability to screen many successive compounds quickly, and obviating the need to apply for ethical approval. This last consideration encompasses significant savings in time and cost for scientists who wish to conduct repellent testing, as there are no volunteers to recruit, enroll, or schedule. Another benefit to repellent testing is the ability to test repellents for duration studies without volunteers. Although field dose rates are typically higher because of the longer duration and potential for abrasive loss and absorption, duration testing of a repellent can be carried out on an in vitro system without the risk of abrasive loss. Similarly, in vitro testing opens up the potential repellent testing with infected vectors, which is currently not possible because of ethical restrictions. However, because the dose of the chemical at which the skin and silicone membrane curves diverge is lower than standard thresholds used in screening these chemicals (1.5 mg/cm^2 or ~7,840 nmol/cm^2), the use of the silicone membrane module system will require further modifications before it can be fully used as a replacement for screening methods using human volunteers.

Acknowledgments

We would like to thank Nathan Newlon for his assistance in drawing the schematics of the modules, and Greg Allen, Maia Tsikolia, and Theresa Cuevas (USDA-ARS-CMAVE) for laboratory technical support with the bioassays. This study was partly supported by the Deployed War-Fighter Protection Research Program and funded by the U.S. Department of Defense through the Armed Forces Pest Management Board.

References Cited

Barnard, D. R., U. R. Bernier, R. Xue, and M. Debboun. 2007. Standard methods for testing mosquito repellents, pp. 103–110. *In* M. Debboun, S. P. Frances and D. Strickman (eds.), Insect repellents: Principles, methods, and uses. CRC Press, Boca Raton, FL.

Bernier, U. R., M. M. Booth, and R. A. Yost. 1999. Analysis of human skin emanations by gas chromatography/mass spectrometry, 1. Thermal desorption of attractants for the yellow fever mosquito (*Aedes aegypti*) from handled glass beads. Anal. Chem. 71: 1–7.

Bernier, U. R., D. L. Kline, D. R. Barnard, C. E. Schreck, and R. A. Yost. 2000. Analysis of human skin emanations by gas chromatography/mass spectrometry, 2. Identification of volatile compounds that are candidate attractants for the yellow fever mosquito (*Aedes aegypti*). Anal. Chem. 72: 747–756.

Bernier, U. R., D. L. Kline, C. Schreck, R. A. Yost, and D. R. Barnard. 2002. Chemical analysis of human skin emanations: Comparison of volatiles from humans that differ in attraction of *Aedes aegypti* (Diptera: Culicidae). J. Am. Mosq. Control 18: 186–195.

Bernier, U. R., K. D. Furman, D. L. Kline, S. A. Allan, and D. R. Barnard. 2005. Comparison of contact and spatial repellency of catnip oil and *N,N*-diethyl-3-methylbenzamide (DEET) against mosquitoes. J. Med. Entomol. 42: 306–311.

Bernier, U. R., D. L. Kline, and K. H. Posey. 2007. Human emanations and related natural compounds that inhibit mosquito host-finding abilities, pp. 77–102. *In* M. Debboun, S. P. Frances and D. Strickman (eds.), Insect repellents: Principles, methods, and uses. CRC Press, Boca Raton, FL.

Butler, J. F., W. R. Hess, R. G. Endris, and K. H. Holscher. 1984. In vitro feeding of Ornithodoros ticks for rearing and assessment of disease transmission, pp 1075–1081. *In* D. A. Griffiths and C. E. Bowman (eds.), Acarology VI, vol. 2. Ellis Horwood, West Sussex.

Cockcroft, A., J. B. Cosgrove, and R. J. Wood. 1998. Comparative repellency of commercial formulations of DEET, permethrin and citronellal against the mosquito *Aedes aegypti*, using a collagen membrane technique compared with human arm tests. Med. Vet. Entomol. 12: 289–294.

Dogan, E. B., and P. A. Rossignol. 1999. An olfactometer for discriminating between attraction, inhibition, and repellency in mosquitoes. (Diptera: Culicidae). J. Med. Entomol. 36: 788–793.

Ellin, R. I., R. L. Farrand, F. W. Oberst, C. L. Crouse, N. B. Billups, W. S. Koon, N. P. Musselman, and F. R. Sidell. 1974. An apparatus for the detected and quantitation of volatile human effluents. J. Chromatog. A 1: 137–152.

EPA. 2000. OPPTS 810.3700: Insect repellents for human skin and outdoor premises. EPA. (https://www.epa.gov/scipoly/sap/meetings/2000/april/insectguid.pdf) (accessed 11 November 2016).

(GraphPad) **GraphPad Software. 2013**. GraphPad Prism version 6.02 for Windows. GraphPad, La Jolla, CA. (www.graphpad.com).

Gratz, N. G. 1999. Emerging and resurging vector-borne diseases. Annu. Rev. Entomol. 44: 51–75.

Karban, R., and J. H. Myers. 1989. Induced plant responses to herbivory. Ann. Rev. Ecol. Syst. 20: 331–348.

Klun, J., 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. Control Assoc. 21: 64–70.

Logan, J. G., M. A. Birkett, S. J. Clark, S. Powers, N. J. Seal, L. J. Wadhams, A. J. Mordue-Luntz, and J. A. Picket. 2008. Identification of human-derived volatile chemicals that interfere with attraction of *Aedes aegypti* mosquitoes. J. Chem. Ecol. 34: 308–322.

Logan, J. G., N. M. Stanczyk, A. Hassanali, J. Kemei, A.E.G. Santana, K.A.L. Ribeiro, J. A. Pickett, and A. J. Mordue-Luntz. 2010. Arm-in-cage testing of natural human-derived mosquito repellents. Malar. J. 9: 239.

Moore, S. J., and M. Debboun. 2007. History of insect repellents. *In* M. Debboun, S. Frances and D. Strickman (eds.), Insect repellents: Principles, methods, and use. CRC Press, Boca Raton, FL.

Mehr, Z. A., L. C. Rutledge, M. D. Buescher, R. K. Gupta, and M. M. Zakaria. 1990. Attraction of mosquitoes to diethyl methylbenzamide and ethyl hexanediol. J. Am. Mosq. Control 6: 469–476.

Posey, K. H., and C. E. Schreck. 1981. An airflow apparatus for selecting female mosquitoes for use in repellent and attraction studies. Mosq. News 41: 566–568.

Roth, A., A. Mercier, C. Lepers, D. Hoy, S. Duituturaga, E. Benyon, and Y. Souares. 2014. Concurrent outbreaks of dengue, chikungunya and Zika virus infections-an unprecedented epidemic wave of mosquito-borne viruses in the Pacific 2012-2014. Euro Surveill. 19: 20929.

Rutledge, L. C., and R. K. Gupta. 2004. Evaluation of an *in vitro* bloodfeeding system for testing mosquito repellents. J. Am. Mosq. Control Assoc. 20: 150–154.

Sastry, S. D., K. T. Buck, J. Janak, M. Dressler, and G. Preti. 1980. Volatiles emitted by humans, pp. 1085–1129. *In* G. R. Waller and O. C. Dermer (eds.), Biochemical applications of mass spectrometry. Wiley, Chichester.

Schreck, C. E., D. L. Kline, and D. A. Carlson. 1990. Mosquito attraction to substances from the skin of different humans. J. Am. Mosq. Control Assoc. 6: 406–410.

(**SAS**) **Statistical Analysis Software Institute. 2012**. Statistical analysis computer software program, version 9.2 for Windows. SAS Institute, Cary, NC.

Weldon, P., J. Aldrich, J. Klun, J. Oliver, and M. Debboun. 2003. Benzoquinones from millipedes deter mosquitoes and elicit self-anointing in capuchin monkeys (*Cebus* spp.). Naturwissenschaften 90: 301–304.