Behavioral insensitivity to DEET in *Aedes aegypti* is a genetically determined trait residing in changes in sensillum function

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***N,N*-Diethyl-*m*-toluamide (DEET) is one of the most effective and commonly used mosquito repellents. However, during laboratory trials a small proportion of mosquitoes are still attracted by human odors despite the presence of DEET. In this study behavioral assays identified *Aedes aegypti* females that were insensitive to DEET, and the selection of either sensitive or insensitive groups of females with males of unknown sensitivity over several generations resulted in two populations with different proportions of insensitive females. Crossing experiments showed the "insensitivity" trait to be dominant. Electroantennography showed a reduced response to DEET in the selected insensitive line compared with the selected sensitive line, and single sensillum recordings identified DEET-sensitive sensilla that were nonresponders in the insensitive line. This study suggests that behavioral insensitivity to DEET in *A. aegypti* is a genetically determined dominant trait and resides in changes in sensillum function.**

One of the most widely used and effective insect repellents available is the synthetic compound *N,N*-Diethyl-*m*-toluamide (DEET) (1). DEET was identified more than 50 years ago by a structure-activity study of synthetic compounds and, although a number of compounds with similar activity have been identified, their efficacy is often judged by comparison with DEET (2). The mode of action of DEET has not been elucidated fully. It was originally thought to act by affecting the olfactory receptor neurons (ORNs) sensitive to lactic acid (3) and thus inhibit the mosquito's response to this, normally attractive, compound (4–6), but this view was challenged by the finding that DEET can function as a repellent even when other attractants are present (4, 7). A recent investigation on *Anopheles gambiae* has suggested that the ORN for 1-octen-3-ol, a component of human sweat (8) that in combination with CO_2 acts as an attractant for this species (9, 10), is blocked by DEET, and thus, in the presence of DEET, a higher concentration of the 1-octen-3-ol is required for the mosquitoes to be able to detect it (11). However, Syed and Leal (12) investigated this theory by using *Culex quinquefasciatus* and suggest that the reduction in response to the 1-octen-3-ol in this species is not because of a diminished response of the ORN but to interactions between the two compounds when DEET and 1-octen-3-ol are tested in the same cartridge. Additionally, single sensillum recordings have identified an ORN that responds directly to DEET in *C. quinquefasciatus* (12) and *Aedes aegypti* (4), indicating that these mosquito species are actively detecting DEET rather than DEET interfering with an ORN detecting another compound.

Despite the proven efficacy of DEET as a repellent, during laboratory and field trials it is common to find that a small proportion of mosquitoes are not repelled by the compound (13–15). Boeckh et al. (4) demonstrated that DEET reduced, but did not entirely eliminate, the approach of *A. aegypti* to host odors, and experiments with *Drosophila melanogaster* (16, 17) and *A. aegypti* (18) have shown the presence of DEET-insensitive individuals. If DEET is detected directly by ORNs, it is likely that any difference in olfactory response to DEET could be detected by changes in the electrophysiological responses of the antenna and in single sensillum recordings from ORNs residing in these sensilla. In *A. aegypti* there are different sets of ORNs present in distinct functional subtypes of the various morphological antennal trichoid sensilla (19, 20), and therefore any change in DEET sensitivity might be detected by changes in recordings from ORNs residing in a specific subtype of these sensilla.

In this study female *A. aegypti* mosquitoes were selected for insensitivity to DEET over several generations, to determine if the trait is heritable. Genetic crosses were then used to establish the mode of inheritance, and electrophysiology to elucidate the neurological basis for the behavioral differences found between DEET-sensitive and DEET-insensitive individuals.

Results

Selection and Crossing. Female *A. aegypti* were tested in a behavioral repellency assay adapted from a World Health Organization test (21) with mosquitoes in a $30 \times 30 \times 30$ cm cage with a 6×12 cm section of metal mesh on top over which an arm with or without DEET was placed. The response of mosquitoes allowed the differentiation between individuals sensitive or insensitive to DEET, where insensitive females would attempt to probe the DEET-treated arm. In the initial laboratory culture, approximately 13% of the females were found to have a DEET-insensitive phenotype and these were crossed with males of unknown sensitivity to establish the "i" line, whereas the females found to be sensitive to DEET were crossed with males of unknown sensitivity to get the "s" line. Two duplicate experiments, A and B, were done. In the F_1 generation of the i line, insensitivity to DEET rose to 50% of females in experiment A and 33% in experiment B (Fig. 1). In both experiments there were significantly more females ($p < 0.001$) probing in the F_1 i line than in either the s line or in the unselected culture. In successive generations the insensitive phenotype plateaued at 53–54% in the i line in both experiments (Fig. 1).

Selection continued up to the F_6 generation in experiment A and the F_5 generation in experiment B, and then the next two generations were reared with no selection, which allowed us to see if the frequency of the trait decreased in the population. The F_9 generation of experiment A and the F_8 generation of experiment B were tested, and there were no further significant changes in the proportion of the population insensitive to DEET

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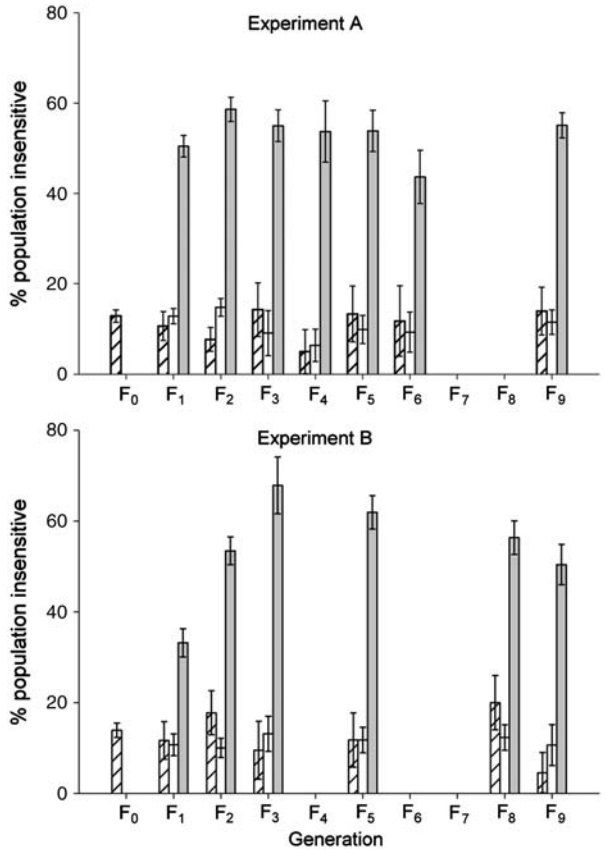

Fig. 1. Percentage of female *A. aegypti* insensitive to DEET in unselected and selected populations in two experiments, A and B. Unselected (dashed bar) = female mosquitoes from standard culture. s (white bar) = line bred from female mosquitoes sensitive to DEET at each generation. i (shaded bar) = line bred from female mosquitoes insensitive to DEET at each generation (F_4 in experiment B was not tested because of low numbers). F_7 and F_8 of experiment A and F_6 and F_7 of experiment B were reared without selection. $N(F_1) = 600$ experiment A, 480 experiment B. $N(F_2–F_9) = 100–400$ per generation. Means are given ±SEM.

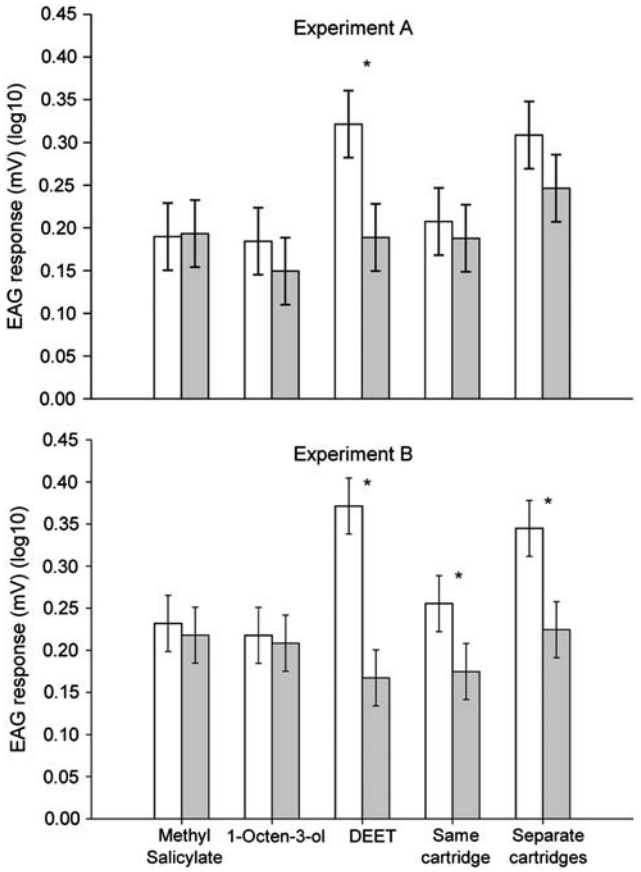

Fig. 2. EAG responses (mV/average control value, data $\log^{10}$ transformed) of *A. aegypti* from the F_9 generation of the s (white bar) and i (shaded bar) lines of experiments A and B. $N = 20$. Same cartridge and separate cartridges refer to DEET and 1-octen-3-ol tested simultaneously by different delivery methods. Concentrations of compounds used: methyl salicylate = 1×10^{-4} g; 1-octen-3-ol = 1×10^{-4} g; DEET = 1×10^{-3} g. * indicates a significant difference ($p < 0.001$). Means are given ± standard error of the difference.

(Fig. 1), which maintained a similar level of insensitivity to previous generations.

Individuals from the F_9 generations of the s lines in experiments A and B were reciprocally crossed with individuals from the i lines, and their offspring were tested for DEET insensitivity, showing a mean percentage insensitivity in the populations between 45% and 55% (Table 1). This insensitivity was not significantly different from the level of insensitivity in the i line ($p = 0.42$).

Electroantennography. Electroantennogram (EAG) recordings were done on 20 female mosquitoes from the s and 20 females from the i lines of the F_9 generations, which had been previously selected in behavioral assays of experiments A and B to determine responses to DEET, (±)1-octen-3-ol, a component of human sweat (8) that has been shown to elicit an electrophysiological effect on recombinant receptors from *A. aegypti* (22), and the plant-derived standard test compound methyl salicylate.

Responses to the control compound methyl salicylate and to 1-octen-3-ol were not significantly different in the s and i lines of either experiment. However, for DEET, in both experiments the i line had a significantly lower ($p < 0.001$) response than did the s line. The results of the EAG experiments are shown in Fig. 2.

DEET was tested in combination with 1-octen-3-ol in the same cartridge, because of evidence that DEET interferes with a mosquito's responses to the 1-octen-3-ol (11). The two compounds were also tested simultaneously, but in separate cartridges, to test for any interaction between the two compounds before they reach the antennae, which would give the appearance of DEET interfering with the 1-octen-3-ol sensitive ORNs when this is not the case (12). In both experiments the s lines showed lower responses to DEET with the 1-octen-3-ol in the same cartridge than when the compounds were in different cartridges (experiment A, $p = 0.002$; experiment B, $p = 0.001$). There were no such differences in the i line. In experiment B there were differences between the responses of the s and i lines to DEET with the 1-octen-3-ol when tested in the same ($p < 0.001$) or different ($p < 0.001$) cartridges, with the i line being lower.

Table 1. Mean percent insensitivity in offspring from reciprocal crosses between *A. aegypti* from selected lines in experiment A and B

Experiment	Parents	Numbers of offspring tested	Mean percent insensitive
A	♀s♂i	118	55
	♀i♂s	118	49
B	♀s♂i	109	45
	♀i♂s	209	50

Single Sensillum Recordings. Single sensillum recordings were done by using female mosquitoes selected by behavioral assay from the

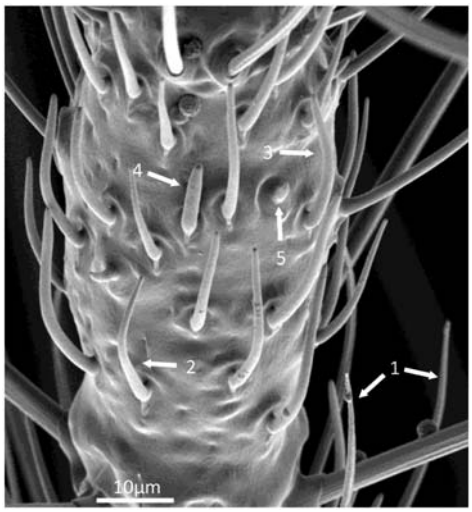

Fig. 3. *A. aegypti* sensilla morphological types: (1) long sharp sensilla; (2) short sharp sensilla; (3) sbtI sensilla; (4) sbtII sensilla; (5) grooved peg sensilla.

s and i lines of experiment A (F_6). Screening all morphological types of trichoid sensilla with a panel of odorants including DEET led to the identification in *A. aegypti* of an ORN that responded to DEET in a dose-dependent fashion in the s line. The ORN was housed in a short blunt type II (sbtII) sensillum (Fig. 3). The sbtII sensilla from both lines were sorted into five functional types according to their responses to compounds excluding DEET, and these functional groups were assigned as subtypes sbtII 1–5 (Fig. 4) according to previous characterizations (19). The compounds chosen had all been reported to have an effect on mosquito behavior or to elicit an electrophysiological response. Linalool and 1,8-cineole are repellent to some mosquito species (23, 24), and thujone has been shown to be attractive to *Culex pipiens* (25). All of these plant-derived compounds affect the DEET-sensitive ORN in *C. quinquefasciatus* (12). 1-Octen-3-ol, which is an attractant for *A. gambiae* in combination with CO_2, was of interest in the mechanism of DEET repellency in *A. gambiae* (11). Indole and acetic acid, found in human sweat (8, 26), were tested because *A. aegypti* trichoid sensilla have been shown to respond to these compounds (19, 27), allowing for the differentiation between functional subtypes.

The responses of the s and i lines were compared in each of the sbtII subtypes. Because there were found to be no type 1 sensilla tested in the i line, type 1 was excluded from comparison. SbtII 1 $N = 5$ s, 0 i. SbtII 2 $N = 2$ s, 5 i. SbtII 3 $N = 2$ s, 1 i. SbtII 4 $N = 7$ s, 4 i. SbtII 5 $N = 7$ s, 1 i. In functional subtypes 1, 2, 3, and 5, the s and i lines showed no response to DEET. In the functional type sbtII 4, the A neuron in the s line showed an excitatory response to DEET at 1×10^{-4} g ($p = 0.04$) and 1×10^{-3} g ($p = 0.048$), and there was a significant difference in the response to DEET between the i and s lines at both concentrations, with the i line having a significantly lower response ($p = 0.007$ and $p = 0.02$, respectively), which was not different from the control ($p = 0.32$ and $p = 0.24$, respectively) (Fig. 5). There was also a significant difference ($p = 0.02$) in the response of this neuron between the two lines to (±)-linalool, with the compound eliciting a significant response from the s line ($p = 0.022$), but the i line response was no different from the control ($p = 0.28$). There was no response in the s or i lines to the (−)-linalool entantiomer. There were no differences in responses within lines between the stereoisomers α-thujone and α,β-thujone.

Discussion

A. aegypti mosquitoes respond differentially to the repellent DEET, with a small proportion of the population being behaviorally insensitive (18). The selection experiments reported here show that breeding from DEET-insensitive females increases the proportion of this phenotype, demonstrating in mosquitoes that the insensitivity is a heritable trait, something seen previously in *D. melanogaster* (16, 17). The rapidity of the increase in insensitivity from 13% to >50% in females between the F_0 and F_2 generations, and the failure to respond to selection after F_3, indicates that the trait is monogenic.

In two separate experiments the proportion of females showing DEET insensitivity plateaued at 53–54% (Fig. 1), and there are at least two possible explanations for this. First, it is possible that the trait is single-locus dominant but homozygous lethal, preventing it from spreading further in the population. However, in the absence of selection a homozygous lethal allele would be expected to fall rapidly in frequency, and this was not seen in the experiments described here when the populations were left unselected for two generations, because the DEET-insensitivity levels were maintained. The second possibility is that all of the mosquitoes in the population have the same genotype that can confer insensitivity to DEET, but that there is incomplete penetrance and so the genotype does not always confer an insensitive phenotype. Incomplete penetrance could result from nonheritable epigenetic differences or environmental factors.

Our crossing experiments with mosquitoes from the s and i lines demonstrated that the insensitive trait is dominant, because the level of insensitivity in the F_1 is indistinguishable from that in the i line and significantly higher than that in the s line. The finding that insensitivity is dominant differs from previous work with *D. melanogaster*, where the trait was found to be either recessive and on the X chromosome (17) or autosomal and partially domi-

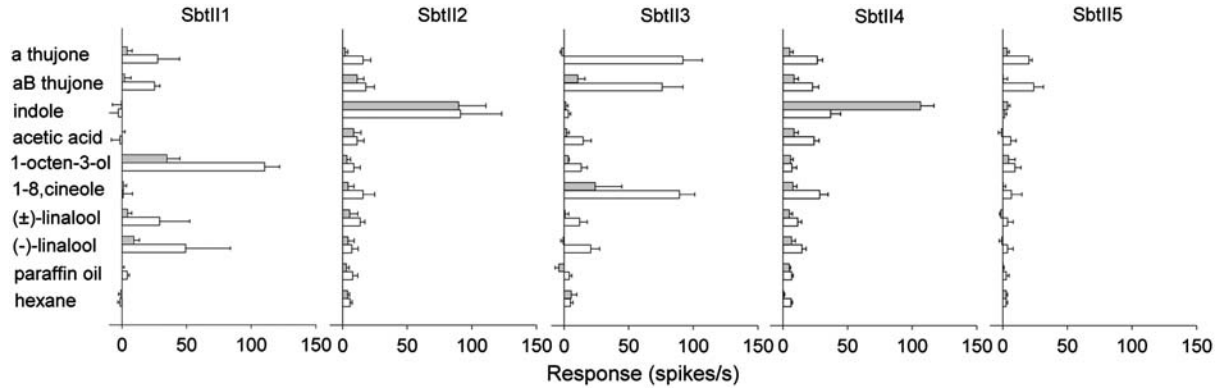

Fig. 4. Response spectra of olfactory receptor neurons housed in sbtII *sensilla trichodea* of *A. aegypti*. The neuronal responses of the two neurons, A (white bar) and B (shaded bar), housed in five functional classes are shown as an average over (*N*) replicates. SbtII 1, $N = 5$ s, 0 i. SbtII 2, $N = 2$ s, 5 i. SbtII 3, $N = 2$ s, 1 i. SbtII 4, $N = 7$ s, 4 i. SbtII 5, $N = 7$ s, 1 i. Means are ±SEM.

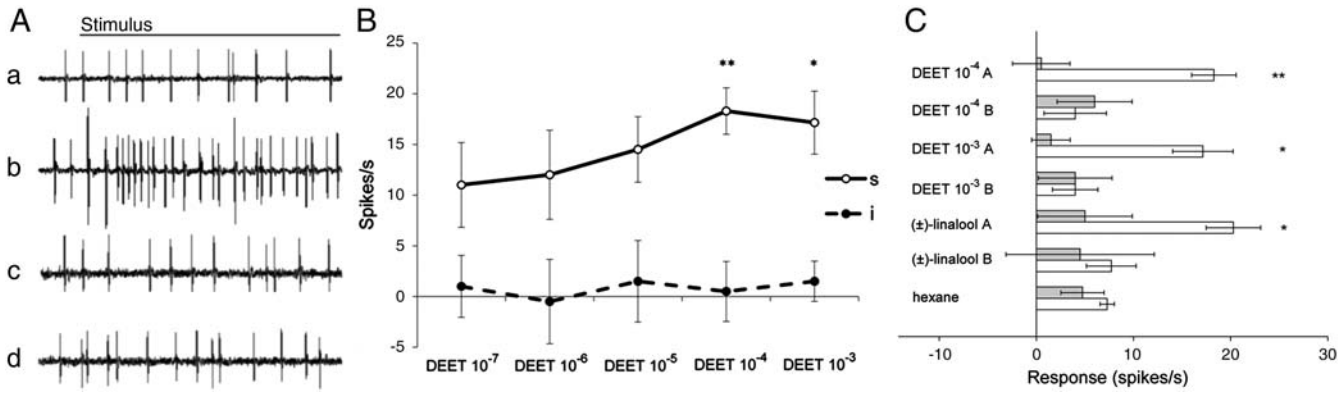

Fig. 5. Responses of sbtII type 4 in behaviorally selected females from the s and i lines. (*A*) Microelectrode recordings from sbtII 4 sensilla. (*a*) s line control. (*b*) s line tested with 1×10^{-3} g DEET. (*B*) Dose–response curve of sbtII 4 to DEET in the s and i lines ($N = 7$ s, 4 i). Here only the responses of the A neuron are shown, because the B neuron showed no response difference to the control. The s line showed an excitatory response to DEET in comparison to the control at 1×10^{-4} g ($p < 0.05$). * indicates a significant difference in response by the s and i lines ($p < 0.05$), ** ($p < 0.01$). (*C*) Neuronal responses to DEET and (±)-linalool by the A and B neurons of sbtII 4 sensilla in female *A. aegypti* from the s (white bar) and i (shaded bar) lines. $N = 7$ s, 4 i. Means are ±SEM. * indicates a significant difference in response by the s and i lines ($p < 0.05$), ** ($p < 0.01$).

nant (16). This difference means that it is likely that the mutations giving rise to DEET insensitivity are in different genes in the two species.

EAG showed that female mosquitoes from the i line had a reduced response to DEET (Fig. 2) in experiments A and B in the F_9 generation. This difference was also shown in the F_5 in experiment A (Fig. S1) and indicates that the behavioral insensitivity is resulting from a change in the mosquitoes' ability to detect the compound at the peripheral olfactory level.

It has been proposed that DEET can act by blocking or interfering with the 1-octen-3-ol ORN in *A. gambiae* (11), so in this study the EAG responses of female mosquitoes were tested in the presence of both DEET and 1-octen-3-ol in the same cartridge. It has also been proposed that DEET prevents the dispersion of 1-octen-3-ol when it is tested in the same cartridge as DEET, as shown in *C. quinquefasciatus* (12), so we also tested the two compounds simultaneously in different cartridges. In experiment B the difference in response to DEET between lines in the presence of 1-octen-3-ol was the same as when tested with DEET alone, regardless of whether the two compounds were supplied in the same or different cartridges, suggesting that DEET does not interfere with the 1-octen-3-ol receptor. This result in *A. aegypti* supports Syed and Leal's (12) conclusions with *C. quinquefasciatus* that DEET does not affect a 1-octen-3-ol sensitive ORN and that these species have an ORN that responds directly to DEET. In experiment A there were no differences in response between lines when tested with 1-octen-3-ol and DEET simultaneously, also suggesting that the behavioral sensitivity is not related to DEET affecting a 1-octen-3-ol ORN. It is possible that in experiment A the presence of 1-octen-3-ol is masking the difference in response to DEET, but why this would be different between experiments A and B is unclear.

By comparing the EAG responses by using the two application methods in the s line, it is clear that there is a significantly lower response to the two compounds tested in the same cartridge than when tested in separate cartridges (Fig. 2). This difference demonstrates in *A. aegypti* that stimulus delivery method can alter the response, supporting Syed and Leal's (12) conclusions with *C. quinquefasciatus*.

Single sensillum recordings have identified an olfactory sensillum type sensitive to DEET in *C. quinquefasciatus* (12), in which the A neuron responds to DEET, α,β-thujone, linalool, and 1-8-cineole and the B neuron to 1-octen-3-ol. In *A. aegypti*, a short blunt sensilla responding to DEET in the A and B neurons has been located (4). In the present study the DEET-sensitive ORN identified in the *A. aegypti* s line was the A neuron and also responded to α-thujone, α,β-thujone, (±)-linalool, 1-8 cineole, indole, and acetic acid, whereas the B neuron responded to indole but not to 1-octen-3-ol. The sbtII 4 sensillum in *A. aegypti* responded to DEET at a lower level than that found by Syed and Leal in *C. quinquefasciatus* (12) but at a similar level to that found by Boeckh et al. in *A. aegypti* (4). It is unclear if the sbtII 4 sensillum found responding to DEET in this study is the same sbt sensillum as found previously in *A. aegypti* (4), where there was no distinction made between sbtI and sbtII morphological types and the functional subtypes were not described. In the Boeckh et al. study both the A and B neurons responded to DEET, whereas in our study only the A neuron in sbtII 4 responds. Thus, we cannot rule out the possibility that we have identified a different DEET-sensitive sensillum. There are likely to be species differences, but it is also possible that the DEET-sensitive sbtII 4 sensillum in *A. aegypti* is not the analogue of the DEET-sensitive sensillum in *C. quinquefasciatus*.

In the present study the sbtII 4 sensilla of the female mosquitoes in the s and i lines responded in the same way to all of the odors tested except for DEET and (±)-linalool (Fig. 4). In each case it was a difference in the response of the A neuron. The A neuron in the i line did not respond to DEET compared to the control. It is therefore likely that the observed reduction in response in the sbtII 4 sensilla accounts for the difference in behavioral response to DEET seen in the insensitive female mosquitoes. With regard to (±)-linalool, the A neuron in the i line responded significantly less than that of the s line. If, as suggested by Syed and Leal (12), DEET is being recognized by a neuron that naturally responds to plant compounds, it is possible that the alteration in the i line that leads to lowered recognition of DEET may also affect the response to the plant-derived compound (±)-linalool. Linalool enantiomers are present in the essential oils of different plants and have distinct scents that insects can differentiate between (28) or that can be detected at different thresholds (29). The ability to detect the enantiomers separately may serve a purpose in the ecology of the insect. In our study, the trait that we selected for was likely to be relevant only to (+)-linalool because the difference between the selected lines occurred only in response to (±)-linalool and not (−)-linalool, with (−)-linalool eliciting no response in either line. The selectivity in response suggests that the enantiomers play a different ecological role, with the ability to detect the (+)-linalool being involved in repellency. It is therefore possible that the insensitivity results from a mutation affecting the way in which DEET is detected. The OR may have been altered so that it can no longer recognize DEET in the same way as it does in the s line mosqui-

toes. Alternatively, there could be a mutation in the gene encoding an odorant-binding protein that normally delivers DEET to the receptor, leading to the odorant-binding protein transporting less DEET to the receptor and thus a lowered response to the compound. Further studies are underway to elucidate the molecular basis for this difference in sensitivity. There is also a need for a study of this kind concerning other compounds with toxic actions such as pyrethroids and dichlorodiphenyltrichloroethane given the rising interest in their use as spatial repellents.

Materials and Methods

Insects. The mosquitoes used in this study were *A. aegypti* [*refm* strain obtained from the Liverpool School of Tropical Medicine (30), replenished with new mosquitoes from Liverpool School of Tropical Medicine in 2007] reared in $30 \times 30 \times 30$ cm Bugdorm 1 cages (Megaview®) in rooms maintained at 27.5 °C ± 1 °C, 60–80% relative humidity, and a 12:12 light:dark cycle. Larvae were reared on Tetramin® tropical fish flakes, and adults were fed on 10% sucrose solution. Females were fed with sheep's blood by using a Hemotek® system. The females used in behavioral experiments were 5–12 days oldand had not been blood-fed. For electrophysiological experiments 7- to 12-day-old females behaviorally selected for insensitivity from the i line, and sensitivity from the s line, were used.

Selection and Crossing. The repellency assay was adapted from a World Health Organization test (21) with mosquitoes in a $30 \times 30 \times 30$ cm cage with clear plastic sides (adapted from Megaview® Bugdorm 1) with a removable 6×12 cm section of metal mesh on top allowing mosquito attraction to an arm to be determined. The experimental room was maintained at 50–70% humidity and 27 °C ± 1 °C. An extractor duct was placed approximately 8 cm from the netting sleeve on the cage with an air flow of 0.18 m/s, drawing air from above the mesh (including volatiles from the arm and the repellent if present) down through the cage and out, preventing buildup of volatile chemicals in the area. Ten female *A. aegypti*, shown to respond to human odor, were placed in each test cage and left to acclimatize for 2 hours, and then a treatment of either 0.5 mL 20% DEET in ethanol or 0.5 mL ethanol alone (control) was applied evenly over the forearm. The ethanol was allowed to evaporate for 30 s, then the arm was placed over the metal mesh at a height of 1.5 cm (preventing the mosquitoes from contacting the arm or DEET), and the behavior of the mosquitoes was observed for 2 min. Mosquitoes that landed on the mesh and attempted to probe the arm were considered to be insensitive to DEET and removed by using a mouth-aspirator into a separate cage, causing as little disturbance as possible to the other mosquitoes. At the end of the 2 min, the mosquitoes that had not landed and probed on the mesh were considered to be sensitive to the repellent. In experiment A, 600 individuals were tested, and 480 in experiment B.

The selected sensitive and insensitive mosquitoes were then used to establish the s and i lines with each generation being tested in the selection bioassay and only the offspring of selected individuals forming the next generation. Unselected culture mosquitoes were also tested for comparison. Mosquitoes were selected in this way for nine generations (the F_4 generation of experiment B was not selected because of low numbers). After several generations of selection, the s and i lines were reared without selection for two generations and then tested with the same procedure above to measure the presence of DEET insensitivity.

Females and males from the F_9 generation were separated into individual containers as the adults emerged and were mated as shown in Table 1. The offspring from each individual cross were then tested in the repellency assay to determine the proportion of offspring insensitive to DEET. Fifty females of each strain in both replicates were crossed, but only 10–16 had enough surviving female offspring to be tested.

Electroantennography. Antennae for EAG were prepared as described by Logan et al. (31). Signals were recorded and analyzed (amplified ×10, 000) on a software package (EAG v2.6, Syntech®, The Netherlands).

The test compound (10 μL in distilled hexane) was applied to a strip of filter paper, and 30 s was allowed for the solvent to evaporate. The filter paper was then placed in a glass pipette cartridge and, by using a stimulus controller, a 2-s air puff was passed through a split airflow system into the continuous airstream through a hole in the glass tube at a 7-cm distance and the response to the stimulus was recorded. The split airflow allowed two compounds to be applied simultaneously. Where there was only one test compound, or when testing the control, the other cartridge contained hexane. Fresh preparations were used for each recording.

Each mosquito was tested with six treatments: a control (hexane), a standard compound (methyl salicylate 1×10^{-4} g), DEET 1×10^{-3} g, (±)-1-octen-3-ol 1×10^{-4} g, and DEET 1×10^{-3} g plus (±)-1-octen-3-ol 1×10^{-4} g in the same cartridge and also in separate cartridges. The control and standard stimuli were applied at the beginning of each test to determine the insect's ability to respond and to establish baseline responses. The control and standard were applied again after every two test treatments. Two minutes were left between each recording. Test treatments were randomized.

Single Sensillum Recordings. A female mosquito was prepared according to the protocol described by Ghaninia et al. (20). A Nikon Eclipse microscope (E600FN) was used to view sensilla on the antenna at high magnification (750×). Single sensillum recordings were performed according to standard protocols described by Stensmyr et al. (32). The ORNs in all morphological types of trichoid sensilla (Fig. 3) were screened for a response to DEET. Once a neuron responding to DEET was located in a sbtII sensillum, further recordings focused on this morphological type. Recordings were made from 23 s sbtII sensilla and 11 i sbtII sensilla with a panel of 9 compounds to determine the functional class of each sensillum and its response to DEET. A DEET dose–response was carried out to ascertain sensitivity. There are two spontaneously active ORNs present in each *A. aegypti* trichoid sensillum, designated A and B (19). Spikes generated by these ORNs were distinguished by shape and amplitude, with the ORN with spikes of higher amplitude classed as A and the ORN with spikes of lower amplitude classed as B. Response to an odor was measured as the difference between the number of spikes for each ORN 0.5 s before and 0.5 s after the stimulus was applied (by using software: AutospikeTM; Syntech) and presented as spikes/s. ORNs were characterized as nonresponding in this study if the response failed to exceed 15 spikes/s. Responses were classified as inhibitory whenever the response was diminished by 10 spikes/s or more.

Because Syed and Leal (12) showed that a DEET-sensitive neuron could respond to α,β-thujone, (−)-linalool, 1,8-cineole, and 1-octen-3-ol, these compounds were tested in the current study, as well as α-thujone and (±)-linalool to see if there were any differences in responses between the stereoisomers of thujone and the (−)-linalool and (±)-linalool. Acetic acid and indole were chosen as additional diagnostic compounds, allowing for differentiation of sbtII subtypes (19). DEET was tested in a dose-dependent manner from 1×10^{-7} to 1×10^{-3} g. All compounds were dissolved in hexane, with the exception of indole, which was dissolved in paraffin oil. Each compound (10 μL) was pipetted onto filter paper (5×20 mm), with 30 s allowed for the solvent to evaporate, and placed in a Pasteur pipette, with 10 μL hexane/paraffin oil used as controls. A stimulus controller (Syntech), was used to deliver a 0.5-s puff from the Pasteur pipette into a hole in the glass tube of the main continuous airflow 10 cm away from the antennae.

Chemicals. α-Thujone (99%), (±)-linalool (95% purity, 70.2% R, 29.8% S), (−)-linalool (98.5%), 1,8-cineole (99%), and acetic acid (99%) were obtained from Fluka. α,β-Thujone (70% α, 10% β), DEET (97%), and indole (99%) were obtained from Aldrich. (±)-1-Octen-3-ol (98%) was obtained from Alfa Aesar.

Statistical Analyses. In the selection trials the proportion of insensitive mosquitoes in different test replicates selected in the F_1 i and s lines were analyzed with a Student *t* test (Genstat®, 11th edition) to determine differences between the lines. The number of F_1 i selected was also compared to the number of insensitive mosquitoes in the laboratory culture. A one-way ANOVA was used to ascertain if there was any difference between the proportion of the i line insensitive to DEET before and after the two-generation gap in selection. Differences were judged significant when the difference between means was greater than the least significant difference (LSD). The proportion of insensitive mosquitoes in the F_9 selected lines and the offspring of crossing experiments were analyzed with a Student *t* test as above.

EAG responses were corrected according to the average of the control values before and after the stimulation of each test treatment. The EAG response in millivolts was divided by the average control value, so that the control value became 1 and the response expressed as a proportion of 1. The data were normally distributed. The mean responses of the two lines to treatments, and between lines for treatments, were compared by using a two-way ANOVA in Genstat® (11th edition), using replicates as blocks. The data were log (base10) transformed. Differences were judged significant when the difference between means was greater than the LSD.

For the single sensillum recordings the response spectra of all ORNs in both the s and i lines were used to classify the sensilla into functional types in a hierarchical cluster analysis. Forty-six s ORNs and 22 i ORNs housed in the sbtII morphological subtype were grouped by using Genstat® (11th edition)

and the group average method according to their responses to a set of eight compounds, and assigned subtypes 1–5 according to previous functional classification (19). Responses to DEET were excluded from the analysis, because these were hypothesized to differ between lines. In each of the five functional groups defined, the s and i line were examined for differences in their responses to compounds including DEET. The first functional group was excluded because none of the sensilla from the i line were in this group. The other four functional groups were analyzed with a two-way ANOVA (Genstat® 11th edition) for line and treatment, by using replicates as blocks, transforming the data with log +25 in order to adjust for negative values. Differences were judged significant when the difference between means was greater than the LSD.

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