Odour receptors and neurons for DEET and new insect repellents

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There are major impediments to finding improved DEET alternatives because the receptors causing olfactory repellency are unknown, and new chemicals require exorbitant costs to determine safety for human use. Here we identify DEET-sensitive neurons in a pit-like structure in the Drosophila melanogaster antenna called the sacculus. They express a highly conserved receptor, Ir40a, and flies in which these neurons are silenced or Ir40a is knocked down lose avoidance to DEET. We used a computational structure–activity screen of ∼400,000 compounds that identified >100 natural compounds as candidate repellents. We tested several and found that most activate Ir40a+ neurons and are repellents for Drosophila. These compounds are also strong repellents for mosquitoes. The candidates contain chemicals that do not dissolve plastic, are affordable and smell mildly like grapes, with three considered safe in human foods. Our findings pave the way to discover new generations of repellents that will help fight deadly insect-borne diseases worldwide.

Blood-feeding insects transmit deadly diseases such as malaria, dengue, lymphatic filariasis and West Nile fever to hundreds of millions of people, causing immense suffering and more than a million deaths every year. Insect repellents can be very effective in reducing disease transmission by blocking contact between blood-seeking insects and humans.

N,N-diethyl-meta-toluamide (DEET) has remained the primary insect repellent used for more than 60 years. However, DEET has little effect on disease control in endemic regions due to high costs and inconvenience of continuous application on skin at high concentrations. DEET also dissolves some plastics, synthetic fabrics and painted surfaces1. Additionally, DEET inhibits mammalian acetylcholinesterase2. Instances of DEET resistance have also been reported in flies3 and mosquitoes4,5. However, the main barriers in developing improved repellents are the estimated cost for identification6 and the subsequent cost of safety analyses for new chemistries.

A significant challenge in finding improved DEET substitutes is that the target receptors through which it repels insects are unknown. Recent studies have given rise to many different models of DEET action. Pure DEET causes inhibition7,8 or mild electrophysiological modification of neural responses to weakly-activating odours in Drosophila antennal olfactory neurons2, but whether these effects contribute to repellency is unknown. Mosquitoes can also directly detect DEET10 and mutations in the co-receptor gene in Aedes aegypti cause reduction in repellency11. Some DEET-sensitive olfactory neurons have been identified in Culex quinquefasciatus12 and A. aegypti13, but it is not yet known whether they are responsible for repellency or which odour receptors they express. A broadly tuned larval odour receptor responds to DEET12,13; however, its role in avoidance in larval or adult mosquitoes has not been demonstrated. Not only can more than one pathway contribute to olfactory repellency, analyses are further confounded by the observation that DEET also activates bitter taste neurons that mediate contact-avoidance in Drosophila14,15.

DEET is detected by neurons of the sacculus

To identify the elusive DEET-sensing neurons of the olfactory system in an unbiased manner, we used the nuclear factor of activated T cells (NFAT)-based system to report DEET-evoked neural activity through expression of green fluorescent protein (GFP) in Drosophila melanogaster16 (Fig. 1a). Exposure to 10% DEET resulted in an increase in expression of GFP in neurons that innervate sensilla within the sacculus, a pit-like structure in the antenna (Fig. 1b, c, Supplementary Fig. 1a and Supplementary Video 1). The dendrites of GFP+ neurons primarily innervated the most distal chamber (I) of the sacculus (Fig. 1c and Supplementary Fig. 1b). Previous studies of DEET overlooked the sacculus because it is intractable to traditional electrophysiology methods. Contrary to expectations from a previous report17, we were unable to find DEET-activated reporter expression in odorant receptor neurons (ORNs) of the maxillary palps (Fig. 1b). We therefore performed single-sensillum electrophysiology analyses and found that the previously reported Or42a+ pb1A neurons responded poorly to DEET, but strongly to hexane that was used as solvent in the previous study (Supplementary Fig. 2a, b).

ORNs innervating the sacculus do not express Or genes, but instead members of a conserved ionotropic receptor (IR) gene family18–21. In the antennal lobes robust DEET–dependent GFP was detected in the characteristic ‘column’ glomerulus (Fig. 1d and Supplementary Fig. 3a), which is innervated by axons of Ir40a-expressing neurons of the sacculus16. Faint GFP was also observed in the Or67d–DA1 glomerulus, which is probably caused by exposure to male pheromone cis-vaccenyl acetate (cVA) in the assay, because the cVA-responsive Or67d+ at1 neuron did not respond to DEET (Supplementary Fig. 2c). The DC4 glomerulus, which is innervated by other sacculus ORNs that express Ir64a+, showed a very faint signal as well (Supplementary Fig. 3a). The simplest interpretation of these results is that Ir40a+ sacculus ORNs innervating chamber I and projecting to the column glomerulus may represent a chief olfactory detection pathway for DEET.

Consistent with previous electrophysiological analyses2,15, we found DEET-dependent GFP expression in gustatory neurons of the labellum...
Moreover, the DEET response is dependent on activation in response to a puff of DEET delivered from an atomizer to express the active form of tetanus toxin (TNTG)24. We blocked synaptic transmission in these neurons using RNAi flies compared to control flies (Fig. 3b). Similar results were obtained when Ir40a-RNAi flies compared to control flies (Fig. 3b). Supplementary Fig. 3b). To test directly whether Ir40a neurons detect DEET and are required for repellency for DEET avoidance when RNAi was induced in Ir40a-Gal4; UAS-GCaMP3/UAS-Ir40a RNAi flies (Fig. 3d). Development using a temperature-sensitive Gal80ts transgene (Fig. 3d). Flies were raised at the permissive temperature (18 °C) until just before adult eclosion, at which point they were left at 18 °C (RNAi off) or shifted to the Gal80ts restrictive temperature 29 °C (RNAi on). Behavioural assays performed four days after the temperature shift showed that Ir40a RNAi in the adult was sufficient to abolish DEET avoidance when RNAi was induced in Ir40a ORNs (Fig. 3e, knockout).

Ir40a is necessary for DEET avoidance

To test directly whether Ir40a is required for olfactory avoidance to DEET, we examined the behaviour of flies in which Ir40a was knocked down pan-neuronally using an elav-Gal4 driver to express a UAS-Ir40a RNA interference (RNAi) construct. In two-choice trap assays (Fig. 3a), we found a significant loss of DEET avoidance in the Ir40a RNAi flies compared to control flies (Fig. 3b). Similar results were obtained when Ir40a RNAi was executed selectively in Ir40a ORNs using two independent UAS-Ir40a RNAi transgenes (Fig. 3c). Not only was avoidance completely abolished, Ir40a knockdown flies actually showed a mild attraction to the DEET trap. Attraction to ACV was unaffected (Supplementary Fig. 4b, c).

We next wanted to rule out the possibility of a developmental role for Ir40a. We therefore suppressed expression of Ir40a-RNAi during development using a temperature-sensitive Gal80ts transgene (Fig. 3d). Flies were raised at the permissive temperature (18 °C) until just before adult eclosion, at which point they were left at 18 °C (RNAi off) or shifted to the Gal80ts restrictive temperature 29 °C (RNAi on). Behavioural assays performed four days after the temperature shift showed that Ir40a RNAi in the adult was sufficient to abolish DEET avoidance when RNAi was induced in Ir40a ORNs (Fig. 3e, knockout).
for the presence of these features. We assembled a training set of known repellents that included: the two commercially approved repellents DEET and picaridin; 34 N-acyl piperidines28 that were identified by structural relatedness to picaridin; natural repellents eucalyptol, linalool, alpha-thujone and beta-thujone10,26,27; and a structurally diverse panel of other odours as negatives28,29. We focused on a descriptor-based computational approach and using a sequential-forward-selection method30 we incrementally identified a unique subset of 18 descriptors that were highly correlated with repellency (correlation of 0.912) (Fig. 4a and Supplementary Table 1). The repellents clustered together if the optimized descriptor subset was used to calculate Euclidean distances amongst odors of the training set (Fig. 4b).

The optimized descriptor set was used to train a support vector machine (SVM), which is a well-known supervised learning approach31, to predict compounds that shared optimized structural features with known repellents (Fig. 4a). A fivefold cross-validation on the training set of repellents was performed and a mean receiver-operating-characteristic (ROC) analysis curve generated. The area under curve (AUC) was determined to be high (0.994), indicating that the in-silico approach was extremely effective at predicting repellents from compounds that were excluded from the training set (Fig. 4c).

We next used the 18-optimized-descriptor and SVM method to screen in silico a large virtual chemical library consisting of >440,000 volatile-like chemicals. Inspection of the top 1,000 predicted repellents (0.23% of hits) revealed a diverse group of chemicals that retain some structural features of the known repellents (Fig. 4d, e). We computed partition coefficient (logP) values of the 1,000 compounds to exclude those predicted to be lipophilic (logP>4.5) and therefore more likely to pass through the skin barrier in topical applications32 (Fig. 4e). We also computed predicted vapour pressures of these chemicals, because volatility may be a useful predictor of spatial volume of repellency (Fig. 4e).

Although the in silico screen was feasible, a more significant challenge lies in identifying safe and effective DEET substitutes that can be rapidly approved for human use. To identify such compounds, we applied our in silico screen to an assembled natural odor library consisting of >3,000 chemicals identified as originating from plants, insects or vertebrate species, and compounds already approved for human use as fragrances, cosmetics or flavours (Supplementary Information). Although many of the top 200 hits share structural features with known repellents from the training set, they also represent structurally diverse chemicals, allowing targeted exploration of previously untested chemical space (Fig. 4f). For example, several antranilates and pyrazines were identified, even though such compounds were largely missing from the training set.

**Ir40a**<sup>+</sup> cells are activated by new repellents

We selected four compounds from the list: methyl N,N-dimethyl anthranilate (MDA), ethyl anthranilate (EA), butyl anthranilate (BA) and 2,3-dimethyl-5-isobutyl pyrazine (DIP), of which the first three have a mild grape-like aroma, excellent safety profiles and have been thoroughly tested and approved for human consumption or oral inhalation by the Food and Drug Administration (FDA), World Health Organization and European Food Safety Authority, and have been listed in the ‘generally recognized as safe’ (GRAS) list by the Flavour and Extract Manufacturer’s Association (Fig. 4g and Supplementary Table 2). The fourth, a pyrazine, is an ant trail pheromone35. The anthranilate and pyrazine classes also contain a large diversity of chemicals found in nature and therefore present attractive repositories of structural substitutes.

For all four chemicals we found robust activation of sacculus ORNs (Fig. 5a, Supplementary Video 2) that innervate the **Ir40a**<sup>+</sup> ‘column’ glomerulus (Fig. 5b, as shown for BA). They also activated gustatory neurons that project to similar areas of the SOG as DEET (Fig. 5b, as shown for BA). GCaMP3 imaging in **Ir40a**<sup>+</sup> neurons showed robust responses to these chemicals, whereas several other classes of common
odorants did not (Fig. 5c and Supplementary Fig. 5). These results demonstrate that the computationally predicted chemicals activate the same chemosensory pathways as DEET and are therefore ideal candidates for new repellents.

In order to test the effect of these compounds on behaviour we used a two-choice trap assay in which flies can sense a DEET-treated filter paper positioned at the entrance of a trap through both olfactory and gustatory systems3,17 (Fig. 5d). All four compounds had strong dose-dependent repellent effects on D. melanogaster (Fig. 5d). Measurements were taken at 24 h and 48 h after the start of the assay, and were found to be consistent. Six additional predicted repellents were tested in a similar manner, at least four of which elicited strong repellency similar to DEET (Supplementary Fig. 6).

To confirm the role of Ir40a-neurons in mediating avoidance to these new repellents, we examined behavioural avoidance of flies in which synaptic activity of Ir40a-neurons was silenced using TNTG1-1 as before. We found that avoidance of chemical treated traps was substantially decreased in Ir40a-TNTG flies as compared to control flies (Fig. 5d), showing that Ir40a-neurons are required for repellency to the four chemicals.

Mosquitoes avoid predicted repellents

To test the effects of the identified chemicals on mosquito behaviour, we adapted an arm-in-cage assay that allows quantitative analysis of chemical repellency on mosquitoes attracted to a human arm. To test the effects of the identified chemicals on mosquito behaviour, we adapted an arm-in-cage assay that allows quantitative analysis of chemical repellency on mosquitoes attracted to a human arm. To test the effects of the identified chemicals on mosquito behaviour, we adapted an arm-in-cage assay that allows quantitative analysis of chemical repellency on mosquitoes attracted to a human arm.

Discussion

The unbiased strategy to use a genetic-reporter of neural activity was instrumental in identifying DEET-sensitive Ir40a-neurons. These reside in the pit-like sacculus that could protect neurons from harsh chemicals. Both olfactory and gustatory systems are activated by DEET, with additional modes of detection in the antenna being mediated by orco12 and a yet to be identified tuning Or gene (Fig. 6h). Additionally, DEET has been reported to have a mild enhancing or suppressing effect on the activity of various Or-expressing...
neurons of antennal basicons in Drosophila, although a causal relationship between this effect and repellency has not been established\(^8\). DEET also has a solvent effect that slows down volatile odour release, potentially also from skin\(^{16}\). Thus, several pathways and mechanisms are likely to participate in overall repellency.

Ir40a can account for the widespread effect of DEET olfactory repellency because it is highly conserved in species that show strong avoidance to it including Drosophila, mosquitoes, head lice\(^\text{34}\) and tribolium\(^\text{35}\), but not in the honey bee\(^\text{36}\). Ir40a orthologues are not as well conserved. The Ir40a-G4/\(^+\); UAS-Gal4/\(^+\) flies exposed to indicated stimuli for 24 h. BA, EA, MDA, DIP are likely to participate in overall repellency.

**Figure 5** Predicted repellents activate Ir40a neurons and are strong repellents for Drosophila. a, Images of antenna of elav-Gal4/\(^+\); LexAop-CD8-GFP-2A-CD8-GFP; UAS-mLexA-VP16- NFAT, LexAop-CD2-GFP/\(^+\);UAS-GCaMP3/\(^+\); UAS-IMPTV/\(^+\); UAS-IMPTV/\(^+\); UAS-TNTG/\(^+\); UAS-TNTG/\(^+\). b, BA, EA, MDA, DIP are likely to participate in overall repellency. c, Mean changes in fluorescence intensity in Ir40a-Gal4/\(^+\); UAS-IMPTV/\(^+\); cells after 2 s application of indicated odors. n = 9–17. d, Mean responses of flies to predicted repellents in two-choice olfactory and gustatory trap assays measured at 24 h and 48 h. n = 3–10 trials (24 h) and 7–10 (48 h); 10 flies per trial, trials with <40% participation were excluded. e, Quantification of flies of indicated genotypes entering repellent-treated traps. n = 6 trials for each genotype, ~20 flies for each trial. P < 0.001, one-way ANOVA with Tukey’s post hoc test. For c–e, error bars represent s.e.m.

**Figure 6** A new class of mosquito repellents with desirable safety profiles. a, Arm-in-cage assay to measure repellency in mosquitoes. b, Mean percentage of female A. aegypti present for >5 s on top net at indicated times to 10% DEET (black line) or solvent controls performed separately (grey line) in a contact (left) or non-contact (right) assay. c, Average time on net for each landing event in b, d. Mean percentage of female A. aegypti present for >5 s on top net in non-contact assay at indicated times. e, Cumulative repellency summed across minutes 2–5 of indicated non-contact treatment (10%) in comparison to appropriate solvent control. Forty mosquitoes were used per trial, n = 5 trials per treatment for b–e. f, Mean weight of vinyl pieces following subersion in indicated compounds or ethanol (control) for indicated amount of time. n = 3, ***P < 10^-5, Student’s t-test. Error bars represent s.e.m. g, Properties of new repellents. h, Model for DEET detection and processing in Drosophila.
to limit insect-human contact in disease-endemic areas of the world and to provide an important line of defence against deadly vector-borne diseases.

**METHODS SUMMARY**

**Physiological experiments.** NFAT-based neural tracing and GCaMP3-based calcium imaging were performed as previously described with some modifications (see Methods). Single-unit recordings from olfactory sensilla were performed as described previously.

**Behavioural experiments.** For olfactory trap assays, 20 *Drosophila* were released in cylindrical arenas containing Eppendorf tube traps (Figs 2d and 3a) with 10% apple cider vinegar as a lure. Repellents were presented on filter papers placed near the traps; flies that entered the trap did not allow physical contact with the fly before its entering the trap. Trap assays to measure repellency when both olfactory and gustatory inputs were possible were performed as described previously. Mosquito arm-in-cage avoidance assays were performed with 40 mated *A. aegypti* females held in a cage and presented a human arm that was inserted in a glove containing a window covered with a double-layer of netting. Test compounds were applied to the nettings. Attraction towards the arm was measured using video recordings and analysts were blind to treatments.

**Chemical informatics.** Optimized molecular descriptors were selected from 3,224 Dragon descriptors based on their ability to increase the correlation between descriptor values and repellency. The repellency-optimized descriptor set was used to first train a support vector machine to predict repellents and then applied to predict new repellents from large compound libraries.

**Insects.** Fly lines were obtained from the Bloomington *Drosophila* Stock Center for TNT and GCaMP3 experiments, the Vienna *Drosophila* RNAi Center for UAS-Ir10a RNAi, J. Wang (UC San Diego) for NFAT tracing, and R. Benton (University of Lausanne) for Ir10a-Gal4. Flies were grown on standard cornmeal-dextrose media, at 25 °C unless otherwise noted and mosquitoes at 27°C and 70% RH.

**Online Content** Any additional Methods, Extended Data display items and Source Data are available in the online version of the paper; references unique to these sections appear only in the online paper.

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METHODS

Fly stocks. Wild-type flies were w1118 backcrossed to Canton-S for 5 generations. UAS-CaMP3 (BL#32326), UAS-TNTG (BL#28838), UAS-LMPTY (BL#28840) and Tub-Pcaul8 (BL#7017) were obtained from the Bloomington Drosophila Stock Center. The following stocks were generously provided: LexAop-CD8-GFP-2A-Cd8-GFP, UAS-mLexA-VP16-NEAT, LexAop-CD2-GFP by J. Wang (UC San Diego, CA), Ir40a-Gal4 by R. Benton (University of Lausanne, Switzerland), and elav-Gal4 by L. Luo (Stanford, CA). UAS-Ir40a RNAi (line 1) (v101725) and UAS-Ir40a RNAi (line 2) (v3960) lines were obtained from the Vienna Drosophila RNAi Center. Ir40a RNAi is predicted to have no off-targets. Fly stocks were grown on standard cornmeal-dextrose media at 25 °C unless otherwise noted. Flies of appropriate genotypes for behaviour experiments were randomly sorted from populations before performing behavioural or electro-physiological experiments.  

NEAT-based neural tracing. Late dark Drosophila pupae ready to emerge of genotype elav-Gal4/ LexAop-CD8-GFP-2A-Cd8-GFP; UAS-mLexA-VP16-NEAT, LexAop-CD2-GFP/+ were collected on moist filter paper strips in culture vials which contained 2 Kimwipes soaked in 5 ml of water in a relatively odour-free environment. A 100 μl sample of odour at the indicated concentration was dissolved in acetone, spread on a filter strip (~1x3 cm), dried for 1 min and placed in a vial with 10–15 pupae. The exposure was given for 24 h and the filter paper strip with odour was replaced at ~12–14 h with fresh odour.  

Calcium imaging using GCaMP3. DEET, DMSO, hexane and candidate compounds were purchased from Sigma-Aldrich or the eMolecules database (http://www.eMolecules.com) from Enamine, Vitas M Labs or Chembridge and were of the highest purity available. Approximately 10–12-day-old flies raised at 29 °C (to improve Gal4 activity) were anesthetized and secured by their wings on double-sided sticky tape (ventral side up) on a Petri dish (BD Falcon, 50 × 9 mm). The fly proboscis, head and body were immobilized by sticky tape as shown (Supplementary Fig. 11). One antenna was stably held down using a glass electrode on thin layer of 70% glycerol that enhanced imaging of fluorescence. The antenna was orientated with the arista and sacculus pointing upwards accessible to odours. Odorants were delivered using 5 ml plastic syringes containing 2 Whatman filter paper strips (2 × 3 cm). A fine mist of DEET at indicated concentration (1%, 0.1% or DMSO) was sprayed into the syringe using an atomizer. Fresh atomized odour syrups were prepared immediately before odour delivery. For DEET substitutes (BA, EA, MDA and DIP), a 100 μl of 50% dilution in DMSO was applied to the filter paper directly and for other odorants 100 μl of 10−2 solution in paraffin or water for apple cider vinegar (ACV) was applied directly on the filter paper. The odour puff (~2 s) was delivered using the syringe over the antenna manually. For imaging odour-evoked activity from the antenna using GCaMP3, a Leica SP5 inverted confocal microscope was used. A filter block with 488 nm excitation filter and 500–535 nm emission filter was used and images were acquired at 3.3 frames per second with a resolution of 330 × 330 pixels using a 10× objective. The settings were optimized to capture odour-induced responses of GCaMP3 with high spatial and temporal resolution while limiting reporter bleaching.  

Data analysis for calcium imaging was performed using the Leica SP5 LAS AF software (in quantize mode) to obtain the heat map images and fluorescence intensity changes. The ΔF/F0 percentage was calculated separately for each selected cell body by taking the mean intensity value of all frames for 5 s before the odorant puff (Fpuff) and taking the mean intensity value of all frames for 5 s around the peak responses (Fpeak) after the end of the ~2 s of stimulus delivery period. Similarly, the mean intensity values were taken for a background area in the vicinity of the cells. The ΔF/F0 percentage was calculated according to the formula:  

\[ \Delta F/F0 = \frac{F_{\text{peak}} - F_{\text{background peak}}}{F_{\text{puff}} - F_{\text{background puff}}} \times 100 \]

Immunohistochemistry. After 24 h exposure to either odour or solvent (control), flies were anesthetized on ice and the tissue dissected in chilled 1× PBS and fixed for 30 min in 4% PFA (0.3% Triton X-100) at room temperature. After washes with PBST (PBS with 0.3% Triton X-100) brains were blocked using PBST with 5% bovine serum albumin (BSA). Rabbit anti-GFP (1:1,000, Invitrogen) and anti-nc82 (1:10 Developmental Studies Hybridoma Bank) were used as primary antibodies and samples were incubated for 3 nights at 4 °C. Alexa Fluor 488 anti-rabbit immunoglobulin G (IgG) (Invitrogen; 1:200) and Alexa Fluor 546 anti-mouse IgG (Invitrogen; 1:200) were used as secondary antibodies, respectively, followed by overnight incubation at 4 °C. Images were acquired with a Zeiss or Leica SP5 confocal microscope and image processing was done using ImageJ and Photoshop software. Data analysis was performed offline, and the investigator was blind to the treatment while counting GFP+ antennal neurons in the confocal micrographs.  

Temperature sensitive Gal80° experiment. For the two-choice behaviour assay in Fig. 3 and Supplementary Fig. 4, flies (10 males and 10 females) with genotypes Ir40a-Gal4/+; UAS-Ir40a RNAi2/Gal80° were grown throughout at 18 °C (permissive temperature) where Gal80 is active and RNAi is off. Such flies were treated as control. In parallel, flies of the same genotype were shifted to 29 °C (non-permissive temperature) from 18 °C as late black pupae for 4 days to activate Gal4 and switch on RNAi. These flies were used as knockdown flies. A subset of flies that were shifted to 29 °C was shifted back to 18 °C for 4 additional days to turn off the RNAi and these were used as recovery flies.  

Electrophysiology. Flies were used 4–7-days-old and raised on cornmeal food at 25 °C. Extracellular recordings were made by inserting a glass electrode into the base of a palp sensillum as done previously29–31. Odorants were diluted in hexane or DMSO, at indicated concentrations (made fresh for every stimulus). For DEET stimulation, 10 μl of diluted odorant was applied to a filter paper strip, the hexane solvent was evaporated for 30 s (or in a previous study32) for 5 min and placed into a glass pasteur pipette cartridge, and each cartridge was only used once. The evaporation of hexane from the filter paper strip was much slower upon mixing with DEET and lingering dampness of the filter paper could be observed visually as well.  

Behavioural testing of Drosophila olfactory avoidance assay for DEET. For each trial, flies that were 3–6 days old (10 males and 10 females) were starved for 18 h. For the trap assay, flies were transferred to a cylindrical 38.1 mm (height) × 84.1 mm (height) chamber containing a trap fashioned from an upturned 1.5 ml microcentrifuge tube with 2 mm removed from the tapered end. A pipette tip (1,000 μl) was cut 2.5 cm from the narrow end and 0.5 cm from top and inserted into the bottom of the inverted microcentrifuge tube. A 15 mm × 16 mm #1 Whatman filter paper was inserted into the pipette tip and tip of microcentrifuge tube so that entering flies could not make physical contact with it. A 25 μl sample of test compound was applied to filter paper and 125 μl of 10% ACV was applied into the upturned lid of the microcentrifuge tube as attractant.  

Trials were run for 24 h and the numbers of flies entering the trap counted (Fig. 2d).  

In the two-choice test, two 10% ACV (125 μl) lured traps as described above were placed in the cylinder, one with 50 μl solvent (DMSO) and another with 50 μl the test odorant at 50% applied to the filter paper (Fig. 3). The more volatile DIP was tested at a lower concentration of 25%. For positive control tests in Supplementary Fig. 4, 125 μl of 10% ACV in test traps and 125 μl of water in control traps was added in the upturned microcentrifuge tube lid. Both traps contained filter papers as before with 50 μl solvent (DMSO). All trials were run for 24 h, positions randomized, and counted. Only trials with >35% participation were considered.  

Preference index = Number of flies in treated trap − number in control trap
Number of flies in treated + control traps (1)  

Drosophila olfactory and gustatory avoidance assay for DEET. Repellency was tested in Fig. 5d and Supplementary Fig. 6 using a Drosophila melanogaster two-choice trap assay as described previously17–19 with minor modifications. Briefly, traps were made with two 1.5 ml microcentrifuge tubes (USA Scientific) and 20 ml pipette tips (USA Scientific), each cap contained standard cornmeal medium. A T-shaped piece of filter paper (Whatman #1) was impregnated with 5 μl of acetone (control) or 5 μl of 10%, 1% or 0.10% test odour, diluted in acetone. Traps were placed in a Petri dish (100 × 15 mm, Fisher) containing 10 ml of 1% agarose to provide moisture. Ten wild-type Canton-S flies 4–7-days-old were used per trial, which lasted 48 h, by which time point nearly all flies in the assays had made a choice. For the 24 h time point data were considered only if >35% of flies had made a choice; at 48 h the majority of flies had made choices. The preference index was calculated as in equation (1) above.  

Mosquito arm-in-cage avoidance assay for DEET. Repellency was tested in mated and starred A. aegypti females using an arm-in-cage assay. A. aegypti mosquitoes (eggs obtained from Benson Research) were maintained at ~27 °C and 70% relative humidity on 14:1:10 light:dark cycle. Behavioural tests were done with 40 mated, non-blood fed, ~24 h starved, 4–10-day-old females in 30 cm × 30 cm × 30 cm cages with a glass top to allow for video recording (Fig. 6a, Supplementary Fig. 7). The experimental protocol was reviewed and approved by the Institutional Review Board (IRB) Compliance Analyst at UCR and does not require additional Human Research Review Board approval. Each test compound solution (500 μl) of 10% concentration in acetone solvent was applied evenly to a white rectangular 7 cm × 6 cm polyester netting (mesh size 26 × 22 holes per square inch) in a glass Petri dish and suspended in the air for 30 min to allow solvent evaporation. The more volatile 2,3-dimethyl-5-isobutyl
pyrazine was dissolved in paraffin oil. Acetone or paraffin oil (500 µl) served as control. A nitrite glove (Sol-velx) was modified as described in Supplementary Fig. 7 such that a 5.8 cm × 5 cm window was present for skin odour exposure. A set of magnetic window frames were designed to secure the treated net ~1.5 mm above skin, and a second untreated netting ~4.5 mm above the treated net in a manner so that mosquitoes were attracted to skin emanations in the open window but unable to contact treated nets with tarsi, or contact and pierce skin. Additionally the test compound had minimal contact with skin. A clean set of glove and magnets were used for every trial. Care was taken that the experimenter did not use cosmetics such as soap on arms. For each trial the arm was first inserted for 5 min and the number of mosquitoes landing or escaping test window recorded on video for a 5-min period. Solvent controls were always tested before a treatment. Mosquitoes showed robust attraction to a solvent treated arm when offered a second time after a gap of 5 mins providing a rigorous test for the treatments to be tested second. No cage was tested more than once within 1 h of a testing session and not more than twice on any single day. Videos were analysed blind and the numbers of mosquitoes present for a 5-s continuous duration were counted every minute. Mosquitoes reliably started accumulating in controls at the 2 min point and data from this time point were considered for analysis.

Percentage present was calculated as the average number of mosquitoes on the window for 5 s at a given time point across trials. All values were normalized to percentage of the highest value for the comparison, which was assigned a 100 per cent present.

Percentage repellency = (1 – (mean cumulative number of mosquitoes on the window of treatment for 5 s at time points 2, 3, 4, 5 min / mean cumulative number of mosquitoes that remained on window of solvent treatment for 5 s at time points 2, 3, 4, 5 min)) × 100.

Escape index = (average number of mosquitoes in treatment that landed yet left the mesh during a five second window over the following time points: 2 min, 3 min, 4 min, 5 min / average number of mosquitoes that landed yet left the mesh during a 5 s window over the same time points in (treatment + control))

For behaviour experiments with preference index, arcsine-transformed data were analysed. Tests used are indicated in the figure legends and they are Student’s t-test, one-way ANOVA and Tukey’s post hoc analysis. Statistical tests for each experimental category and sample trials sizes were selected on the basis of previously published studies using similar assays, which are cited throughout the manuscript. For all graphs, error bars indicate s.e.m.

Chemical Informatics. A single energy-minimized three-dimensional structure was predicted for each compound using the Omega2 software package46. The commercially available software package Dragon (3,224 individual descriptors) from Talete was used to calculate molecular descriptors47. Descriptor values were normalized across compounds to standard scores by subtracting the mean value for each descriptor type and dividing by the standard deviation. Molecular descriptors that did not show variation across compounds were removed.

For our analysis, compounds from different studies were approximated into a single metric of ‘protection duration’ as a rough indicator of repellency. The non-repellent diversifying training set of odours were assigned protection times of zero, whereas the approved repellents DEET and picaridin were assigned the highest value since we made the assumption that these would have structural properties important for regulatory approval. Compounds were clustered using Euclidean distance and hierarchical clustering based on differences in repellency values, and a set of 5 compounds with the highest activity that clustered together was classified as ‘training repellents’.

A compound-by-compound repellency distance matrix was calculated from repellency data. A separate compound-by-compound descriptor distance matrix was calculated using the 3,224 descriptor values calculated by the Dragon software package. Using a sequential forward selection (SFS) approach, all descriptors are individually compared and selected for their ability to increase the correlation between descriptor values and repellency. The descriptor that correlates best is retained and each further iteration adds an additional descriptor to improve the correlation values. This process is continued until additional descriptors fail to improve the correlation value from the previous step. This process results in a unique descriptor set that is optimized for repellency.

This repellency-optimized descriptor set was used to train a support vector machine (SVM) using regression and a radial basis function kernel available in the R package e1071, which integrates libsvm48. Optimal gamma and cost values were determined using the tuneSVM function. The resulting trained SVM was then applied to predict activity for compounds from two libraries in silico, a natural compound library of ~3,200 volatiles and a > 440,000 compounds library.

For the natural compound library we assembled a subset of 3,197 volatile compounds from defined origins including plants, humans, insects49, food flavours and a fragrance collection50 including fruit and floral volatiles49–51. For the larger library we assembled a subset of ~400,000 small molecules from the eMolecules database that have properties of volatile odorants. (Molecular weight <325 grams per mole and atoms: C, O, N, H, S.)

We performed a fivefold cross-validation by dividing the data set randomly into five equal sized partitions. Four of the partitions were applied to train the SVM and the remaining partition, which was not used for training, was used to test predictive ability. This process was repeated five times, each trial excluding a different subset of compounds as the training set and assigning the remainder as the test set. The whole process was repeated 20 times to improve consistency. A receiver operating characteristics (ROC) analysis was then used to analyse the performance of our computational repellency prediction. The overall predictive ability was calculated as a single receiver operating characteristic (ROC) curve for all 20 independent validations.

Calculation of LogP and vapour pressure values. SMILES structures of the predicted repellent odours were used with EPI Suite (http://www.epa.gov/optpt/exposure/pubs/epsuite.htm) to calculate predicted LogP and vapour pressure values.

Vinyl solubility test. One 3 × 3 mm square of 4 gauge vinyl was submerged in 1 ml of each test compound in a glass container, stirred at a constant rate on a shaker and checked every 30 min until the vinyl square in DEET was completely dissolved (6 h). The vinyl pieces in each of the other compounds were removed, rinsed in ethanol and weighed. The process was repeated at 30 h (24 h after the vinyl square completely disappeared in DEET).

Statistical analyses. For behaviour experiments with preference index, arcsine-transformed data were analysed. Tests used are indicated in the figure legends and they are Student’s t-test, one-way ANOVA and Tukey’s post hoc analysis. Statistical tests for each experimental category and sample trials sizes were selected on the basis of previously published studies using similar assays, which are cited throughout the manuscript. For all graphs, error bars indicate s.e.m.
Retraction: Odour receptors and neurons for DEET and new insect repellents

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We are retracting this Article because we no longer have confidence in data that support one of our key conclusions. In this Article we reported four advances in insect repellency: identification of olfactory neurons in Drosophila melanogaster that participate in repellency to N,N-diethyl-meta-toluamide (DEET); identification of an ionotropic receptor, Ir40a, expressed in these neurons required for avoidance to DEET; development of a chemical informatics method of identifying shared structural features from known behavioural repellents; and validation of a series of computationally identified natural chemicals as repellents for flies and mosquitoes. We no longer have confidence in data supporting that Ir40a is a DEET receptor. Upon reanalysis, the original calcium imaging (GCAMP) data show movement artefacts and background effects that we originally missed, which seriously undermine our confidence in Ir40a responses to DEET. In addition, Supplementary Fig. 5b presents several inappropriately re-used panels.

Upon learning that A. F. Silbering et al. did not find defects in DEET aversion in Ir40a mutant flies, we repeated many of the original behaviour experiments. Although we confirmed significant behavioural differences in Ir40a cell-silenced flies (Ir40a-Gal4;UAS-TNTG), as reported in Fig. 2d, we have been unable to replicate observations of behavioural experiments using Ir40a-Gal4;UAS-RNAi flies. Therefore, with the exception of author Pinky Kain, we no longer have confidence in the conclusions of Figs 2, 3 and 5c, and Supplementary Fig. 5. We remain confident of the chemical informatics analyses and the identification of new repellents, which have been successfully repeated in our laboratory and by others, as reported in Figs 4, 5d and e, 6, and Supplementary Figs 2 and 6–9. Although it may still be possible that Ir40a does respond to DEET, given the issues listed above, all authors except Pinky Kain wish to retract this Article in its entirety. We deeply regret these circumstances and apologize to the scientific community.

1. Silbering, A. F. et al. Ir40a neurons are not DEET detectors. Nature 534, E5–E7, http://dx.doi.org/10.1038/nature18321 (2016).