The oviposition cue indole inhibits animal-host attraction in *Aedes aegypti* (Diptera: Culicidae) mosquitoes

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Abstract

Background

Mosquitoes represent a major source of disease transmission worldwide. They possess the uncanny ability to discriminate between different ecological resources, including nectar sources, animal-hosts, and oviposition sites, a feature mediated by their exquisite olfactory system. Insect repellents such as N,N-Diethyl-meta-toluamide, also called DEET, have been shown to activate and inhibit mosquito odorant receptors, resulting in behavioral modulation. This and other repellents available for personal protection against mosquitoes are topically applied on the skin and operate at a short range. In our search for potential long-range inhibitors of human-host attractants, we have hypothesized that the shared chemical similarities between indole and DEET may confer the former the ability to block odorant receptor function and inhibit human-host attraction.

Methods

We used the two-electrode voltage clamp of *Xenopus laevis* oocytes as a pharmacological platform, to compare the pharmacological effect of commercially-available insect repellents and indole on the *Aedes aegypti* (*R*)-1-octen-3-ol receptor OR8, a receptor involved in the decision of female mosquitoes to identify human hosts. We conducted an arm-in-a-cage and a wind-tunnel bioassays to explore the effect of indole on human-host seeking female *Aedes aegypti* mosquitoes.

Results

We provide evidence that indole inhibits the *Aedes aegypti* (*R*)-1-octen-3-ol receptor OR8, a receptor involved in the decision of female mosquitoes to identify human hosts. In our arm-in-a-cage assay, one molar DEET reduced mosquito visits on average by 69.3% while the same indole concentration achieved 97.8% inhibition. This effect of indole on flight visits was dose-dependent and disappeared at one micromolar. In our long-range bioassay, indole elicited on average 27.5% lower speed, 42.3% lower upwind velocity and 30.4% higher tortuosity compared to our synthetic blend.

Conclusions

Indole significantly inhibits OR8 activation by (*R*)-1-octen-3-ol, mosquito visits to a human hand, and long-range human-host seeking. The volatility of indole may be leveraged to develop a novel insect repellent in the context of personal mosquito.

Introduction

Mosquitoes are a major source of infectious diseases worldwide due to their ability to transmit pathogens and are also a major source of annoyance. While N,N-Diethyl-meta-toluamide (DEET) and other topical repellents provide sufficient personal protection to ward off these insects, these compounds act at a short range [1–3]. Despite the protection repellents provide, mosquitoes are still able to efficiently locate potential human hosts, hover around them and locate any unprotected area that can be targeted for biting. It is therefore desirable to find alternative solutions with extended protection range such as volatile odorant repellents [1]. Unlike topical repellents, odorants such as geraniol, citral, citronellal, eugenol, and anisaldehyde have been shown to exhibit various degrees of spatial repellency against mosquitoes (Hao et al. 2008). In the future, these plant volatiles may play a major role in the development of spatial repellent technology.

Mosquito repellents such as DEET or Insect Repellent 3535 (IR3535) exhibit low volatility and act at a short range. These compounds exhibit complex interactions with odorant receptors (ORs), activating some ORs while inhibiting others [4–6]. Notwithstanding its odor masking properties [7–9], DEET alone activates the *Ae. aegypti* indole receptor AegOR2 and also inhibits (*R*)-1-octen-3-ol-activated AegOR8 [4]. The *OR8* gene is expressed in basiconic sensilla on the maxillary palps of mosquitoes belonging to species of the Anopheline and Culicine families [10, 11]. This phylogenetically-conserved receptor specifically responds to the (*R*)-1-octen-3-ol enantiomer in the nanomolar range [12, 13] and is believed to synergize the effect of CO₂ in the attraction to human-hosts [14–16].

Indole alone does not mediate oviposition in *Anopheles gambiae* (Diptera: Culicidae) [17] and *Ae. aegypti* [18] unless combined with 3-methyl-1-butanol [17] in *An. gambiae*, and nonanal, p-cresol, phenol and dimethyltrisulfide in *Culex spp.* (Diptera: Culicidae) [19]. High indole dosages have been demonstrated to repel the oviposition of mosquitoes such as *Toxorhynchites spp.* (Diptera: Culicidae) [20], *Cx. quinquefasciatus* [21], *Aedes spp.* [18] and *An. gambiae* [22].

Noting that DEET and indole exhibit overlapping chemical features, including the presence of an aromatic ring and a neighboring nitrogen atom (Fig. 1A), we surmised that indole might interfere with OR8 function. Using the two-electrode voltage clamp of *Xenopus laevis* oocytes expressing *Ae. aegypti* *OR8*, we show that indole inhibits OR8-mediated current depolarizations elicited by (*R*)-1-octen-3-ol. We also show that sustained exposures to indole and repellents generate an increase of the current baseline. We observed that co-administration of indole and (*R*)-1-octen-3-ol evokes significant hyperpolarizations that are concentration-dependent. In light of these pharmacological results, we tested the potential behavioral effect of indole on animal-host-seeking female *Aedes aegypti* using an arm-in-a-cage assay, and provide evidence that indole acts as a repellent at various doses. Using a wind tunnel, we show that indole decreases anemotactic flight elicited by a synthetic human host blend composed of CO₂ and 1-octen-3-ol. Our findings suggest that volatile compounds such as indole may be discovered based on the pharmacological knowledge of ORs. The volatility of indole and the phylogenetic conservation of the CO₂ and octenol receptors in the Culicidae family may be leveraged to develop a novel insect repellent with a broad spectrum of action in the context of personal mosquito protection.

Methods

Aedes aegypti mosquitoes

The mosquito laboratory-reared colony developed by Prof. Joel Margalit is maintained in an air-controlled insect chamber with a temperature of 26°C, 80% relative humidity, and 12:12h light/dark photoperiod. Mosquito larvae were kept in plastic pans containing one liter of water. Larvae were fed with a ground mixture of Novocrabs feed (JBL GmbH & Co, Germany), mice feed and dry yeast until adult emergence. Pupae were transferred into plastic cages and eclosed adults were allowed to mate and feed *ad libitum* with a 10% sucrose solution. Females were fed with cow blood using a membrane feeding system.

AegOR8-Orco mRNA expression in *Xenopus laevis* oocytes

In vitro transcription and two-microelectrode voltage-clamp electrophysiological recordings were carried out as previously described (Dekel et al., 2016). Both AegOR8 and AegOrco were synthesized using the mMESSAGE mMACHINE® SP6 kit (ThermoFisher Scientific) from the linearized pSP64tRFA expression vector. *X. laevis* oocytes were manually collected, separated, and treated for 30 min with a 8mg/mL collagenase solution at 18°C to remove the follicular layer. Stage V-VI oocytes were rinsed in Ringer solution (96 mM NaCl, 2mM KCl, 5mM MgCl₂ and 5mM HEPES, pH 7.6) and microinjected with a mixture of 1 µL AegOR2 (3 µg/µL), 1 µL AegOrco (3 µg/µL) and 1 µL of double-distilled water. Injected oocytes were maintained at 18°C for 3 days in Ringer's solution (96 mM NaCl, 2 mM KCl, 5 mM MgCl₂, 0.8 mM CaCl₂ and 5 mM HEPES, pH 7.6) supplemented with 5% dialyzed horse serum, 50 µg/mL tetracycline, 100 µg/mL streptomycin and 550 µg/mL sodium pyruvate.

Whole-cell currents were monitored and recorded using the two-microelectrode voltage-clamp technique. Holding potential was maintained at -80 mV using an OC-725C oocyte clamp (Warner Instruments, LLC, Hamden, CT, USA). Oocytes were placed in a RC-3Z oocyte recording chamber (Warner Instruments, LLC, Hamden, CT, USA) and exposed for 8 seconds to different concentrations of indole (CAS 120-72-9, Sigma-Aldrich), (*R*)-1-octen-3-ol (CAS 3687-48-7, 98.2%, Bedoukian Research Inc.), N,N-Diethyl-m-toluamide (DEET; CAS number 134-62-3, Sigma-Aldrich), and IR3535 (CAS 52304-36-6, Merck). All compounds were solubilized in 200 µL of dimethyl sulfoxide (DMSO) prior to dilutions in Ringer's buffer. *Orco* alone was expressed in oocytes and exposed to *N*-(4-Ethylphenyl)-2-[[4-ethyl-5-(pyridin-3-yl)-4*H*-1,2,4-triazol-3-yl]sulfanyl]acetamide (VUAA1, from Innovapharm Ltd) as a positive control. Currents were allowed to return to baseline between odorant applications. Data acquisition were carried out with a Digidata 1550A and pCLAMP10 (Molecular Devices, Sunnyvale, CA, USA). Plotting and statistical analyses of the data were performed using GraphPad Prism 7 (GraphPad Software Inc., La Jolla, CA, USA).

Human-host seeking arm-in-cage bioassay

To investigate the potential role of indole in human host-seeking behavior, we developed an arm-in-cage bioassay in which the experimenter's hand is presented to fifteen 5–10-day-old post emergence adult females. The bioassay is comprised of a custom-designed 3D-printed interlocking ring that creates a 55

mm diameter opening in a powder free latex glove worn by the experimenter. A plastic screen is wedged between the two ring components to protect against mosquito bites. The ring supports an odorant delivery platform located at the center of the ring, which receives a 10 mm diameter cover glass and two 5 mm diameter filter discs (WHA10016508, Merck) (Additional file 1: Fig. S1.; Additional files 2&3). Mosquitoes are introduced in a 20.3 cm³ metal cage located inside an incubator for at least 10 minutes before each experiment (26°C, 80% RH) for acclimatization. The cage is then placed at room temperature of (23–25 °C) under a video camera (Canon, EOS 70D, lance: MACRO 0.25/0.8ft) to monitor and quantify the numbers of mosquito visits and their duration. On a blank filter discs, 25 µL of the solvent diethyl ether (vehicle), indole or DEET were deposited on the filter paper and allowed to evaporate for 2 minutes prior to mosquito exposure. We used 10-fold dilutions of indole ranging from 10⁻⁶-10⁻¹ M, and 10⁻¹ M DEET. The experimenter rubbed the ring-mounted glove against the shirt and skin. All experiments were conducted between 10:00 and 13:00 and lasted 10 minutes. This schedule was chosen for practical reasons and also because mosquitoes consistently exhibited attraction to the human hand. To determine the repellency of indole against human-host seeking mosquitoes, we recorded the number of females landing per minute and counted the duration of their stay on the screen surface. Mosquito landing count was normalized ($x' = \frac{x - \min(x)}{\max(x) - \min(x)} * 100$). Mosquitoes returning to the region of interest were counted as a new visit. Statistical comparisons of mosquito landing were carried out with a Kruskal-Wallis H test and post-hoc pairwise comparisons using Wilcoxon rank sum exact test (*P* value adjustment method: Benjamini-Hochberg (BH)). A one-way ANOVA analysis was used to analyze mosquito duration.

Wind tunnel bioassay

A wind tunnel system was used to measure the response of female *Ae. aegypti* to CO₂ alone, 1-octen-3-ol and indole stimuli. The wind tunnel is a rectangularly-shaped chamber (2200 x 500 x 500 mm), which is composed of three compartments, including release, flight, and odor delivery chambers. The release chamber holds the mosquitoes prior to the experiment, while the odorant chamber releases odor from a clean air delivery system (Sigma Scientific LLC., USA). Ten females (same age as previously described) were transferred to a release box (200 x 160 x 200 mm) inside the release chamber (500 mm x 200 mm x 500 mm) and allowed to acclimate for 1 hour in darkness with 27 ± 0.7°C. Experiments were conducted between 10:00 and 13:00 local time. Three experiments were conducted for each treatment. The flight tunnel (1500 x 500 x 500 mm) was illuminated with 5 infra-red lights (RT VAR2-i2-1, Raytec Ltd., United Kingdom) and a laminar air flow was applied at a speed of 0.1 m/s. After acclimatization, the injection air flow was manually opened for 15 min (duration of the experiments) with compressed air (4 L/m), which delivered the stimulus. Immediately afterwards, flight coordinates were recorded using TrackIt (SciTrackS GmbH, Switzerland) and the release box door opened. The time interval between each experiment was at least 30 min under constant room ventilation with the wind tunnel top cover removed. Manual maintenance of the wind tunnel was carried out using nitril latex free gloves to prevent odor contamination.

Tracking software and data analysis

Flight trajectories were recorded using two acA2000-165um infrared-sensitive cameras (Basler AG, Germany) and processed by the 3D software TrackIt (SciTrackS GmbH, Switzerland). Output data was analyzed through R version 4.0.2 programming language [23]. A description of the functions to quantify parameters of flight trajectories can be found in Additional file 4: Table S1. In total, the combined treatments consisted of 90614 raw X,Y,Z coordinates. Coordinates located outside the flight tunnel (reflection) were removed. The visual field was cropped to define a 800 x 500 x 500 region of interest (ROI, Fig. 5A). Coordinates within the first three minutes of recording were retained and analyzed. We randomly sampled 25% of the remaining coordinates (5192 coordinates) for statistical analysis. Statistical comparisons between treatments were carried out with a Kruskal-Wallis H test and post-hoc pairwise comparisons using Wilcoxon rank sum exact test (*P* value adjustment method: Bonferroni).

Odorant stimuli

The injected stimuli included (i) CO₂ (600 ± 20 ppm) + 25 µL of DEE, (ii) CO₂ + 25 µL of DEE + 2 µL of 1-octen-3-ol (168 mg), and (iii) CO₂ + 25 µL of DEE + 2 µL of 1-octen-3-ol (168 mg) with indole 5 M (29 mg). Each stimulus was deposited on a double Whatman filter paper (WHA10016508, Merck) as described above in the arm-in-cage bioassay. To allow DEE to evaporate, the holder was placed for 2 minutes in a separate room. Afterwards, the chemical holder was placed inside an OSI-4550 inline volatile collection chamber (Sigma Scientific LLC., USA) connected between the clean air delivery system and the injector. Carbon dioxide was delivered by a steel compressed gas cylinder. The CO₂ content was monitored at the injector site using a TES-1370 CO₂ analyser (TES electrical electronic Corp., Taiwan). During all these procedures, the experimenter wore laboratory gloves and a mask.

Results

Indole inhibits AaegOR8 activation by (*R*)-1-octen-3-ol.

Based on the chemical similarities between indole and DEET (Fig. 1A), we surmised that indole exerts an inhibitory effect on AaegOR8. A 10⁻³ M indole concentration reduced by approximately 30% the AaegOR8 current amplitude elicited by 10⁻⁷ M (*R*)-1-octen-3-ol (Fig. 1B). This effect was not observed in *Orco*-injected oocytes.

To determine the nature of this inhibitory effect, we established a series of concentration-response curves using OR8-Orco-injected oocytes exposed to increasing concentrations of (*R*)-1-octen-3-ol alone or combined with 10⁻⁴ M, 10⁻³ M or 10⁻² M indole (Fig. 1C). Two types of currents were observed, including the expected agonist-induced depolarization currents and unusual indole-dependent hyperpolarization or reduction in baseline currents.

The interpolated EC₅₀ values for octenol alone versus 10⁻⁴ M and 10⁻³ M indole were not statistically significant (Fig. 1D, Additional file 5: Table S2). The EC₅₀ value elicited by 10⁻² M indole was significantly different from (*R*)-1-octen-3-ol alone but was moderate. These findings suggest that indole does not have

an important effect on the sensitivity of this receptor for (*R*)-1-octen-3-ol. Contrary to our initial results (Fig. 1B), we did not observe any significant inhibition of the amplitude response as the indole concentration increased (Fig. 1E). However, current amplitudes are contingent on oocyte inherent variability. To address this limitation, we determined the systematic effect of indole on depolarization current amplitudes by normalizing all the current responses to the initial 10^{-7} M (*R*)-1-octen-3-ol exposure (Fig. 1C). 10^{-2} M indole consistently reduced the current amplitude across the concentration of (*R*)-1-octen-3-ol, excluding 10^{-10} M. We confirmed that 10^{-3} M indole significantly reduced the response amplitude of AegOR8 to 10^{-7} M (*R*)-1-octen-3-ol (Fig. 1F).

Indole concentration of 10^{-3} M and 10^{-2} M elicited hyperpolarization currents (reductions in current baseline) in the presence of (*R*)-1-octen-3-ol concentrations ranging from 10^{-10} M to 10^{-7} M (Fig. 1C). These hyperpolarization currents were concentration-dependent and were surmounted at higher (*R*)-1-octen-3-ol concentrations (Fig. 1G). To understand the contribution of indole alone to these currents, we exposed OR8 to increasing concentrations of indole (Fig. 2A). 10^{-6} to 10^{-4} M indole evoked small depolarization currents. The two highest indole concentrations elicited either depolarization or hyperpolarization currents that were a fraction of the initial 10^{-7} M (*R*)-1-octen-3-ol stimulation. To better characterize these small yet inconsistent effects, we focused on the currents elicited by 10^{-2} M indole and consistently observed these small hyperpolarization and hyperpolarization currents (Fig. 2B). By comparison, this same concentration of indole, in the presence of (*R*)-1-octen-3-ol at concentrations as low as 10^{-10} M, elicited currents reaching the initial response to 10^{-7} M (*R*)-1-octen-3-ol, suggesting that the significant indole-induced hyperpolarization currents require the presence of (*R*)-1-octen-3-ol (Fig. 2C).

Indole modifies the OR8-mediated current baseline of the oocyte membrane.

The cause for the observed hyperpolarization currents caused by indole in the presence of (*R*)-1-octen-3-ol was intriguing. It mirrored a phenomenon previously documented with AegOR8, AegOR2 and AegOR10 [4,6] and more recently with additional ORs from *Culex quinquefasciatus*, *Aedes aegypti*, and *Anopheles gambiae* [24]. To investigate whether the hyperpolarization current was a transient response or a durable modification of the current baseline, we exposed OR8-injected oocytes to a change of perfusion buffer by switching from the ND96 solution to a $5 \cdot 10^{-3}$ M indole perfusion. We also administered increasing ten-fold dilutions of (*R*)-1-octen-3-ol before reversing the perfusion solution back to ND96 (Fig. 3A). Prior and after the two perfusion buffer exchanges, the oocyte was exposed with a transient stimulation of 10^{-7} M (*R*)-1-octen-3-ol for control purposes. The switch from ND96 to indole elicited a stable decrease in the baseline current not observed in water-injected oocytes (Fig. 3A). (*R*)-1-Octen-3-ol produced very little depolarization currents at all tested concentrations. By

comparison, DEET and IR3535 evoked larger currents. The opposite buffer switch exhibited a stable decrease in baseline current most pronounced in the case of DEET and IR3535 as well (Fig. 3A). In these experiments, we treated the oocytes with a lower indole concentration, as compared to DEET and IR3535,

because 10^{-2} M indole consistently killed the perfused oocytes. All observed currents elicited by (*R*)-1-Octen-3-ol, indole, a mixture of these two ligands, and buffer switch are summarized in Fig. 3B.

Indole inhibits close-range human-host attraction.

To explore the behavioral role of indole, we exposed a human hand to female mosquitoes using an arm-in-a-cage assay (Fig. 4A insert). The hand was covered with a protective glove allowing mosquitoes to detect human skin odor through a window created by an open area on the dorsal side of the hand (Additional file 6: Video S1). This open area was protected by a screen and was equipped with an odor delivery system (Additional file 1: Fig. S1., Additional files 2&3). Increasing doses of indole ranging from 10^{-6} to 10^{-1} M were deposited on this delivery system and repellency was measured in terms of number of mosquito visits and duration of visits. The repellency effect of DEET was significantly different from vehicle and indole 10^{-6} M (Fig. 4A) (Kruskal-Wallis H test, $H = 106.11$, $df = 8$, $p\text{-value} < 0.0001$). All indole treatments, except 10^{-6} M, were significantly different from the vehicle (Fig. 4A). Increasing indole doses reduced the number of mosquito visits from 40.6 to 93.8%. We observed a 3.6% inhibition with a 10^{-6} M indole concentration but this effect was not statistically significant. Looking at the accumulated landing numbers, vehicle and 10^{-6} M indole elicited overlapping temporal dynamic (Fig. 4B). Indole at 10^{-1} M had a significantly higher temporal repellency than all other treatments including DEET at the same concentration. Other indole treatments exhibited intermediate temporal repellency between these two extremes. However, the only significant differences were observed between the vehicle and 1M indole (Additional file 7: Fig. S2). In terms of visit durations, mosquitoes spent on average the same amount of time on the open area when landing occurred (Fig. 4C) (ANOVA, $F_{(8,18)} = 1.219$, $P = 0.343$).

Indole reduces 1-octen-3-ol-mediated attraction.

While we have circumstantial pharmacological evidence that indole may in part affect OR8-mediated detection, we do not have any direct indication that indole affects 1-octen-3-ol-mediated attraction in the context of human host-seeking. To explore this possibility, we used a flight tunnel (Fig. 5A) to expose human-host seeking female mosquitoes to a synthetic blend composed of CO_2 and 1-octen-3-ol. We used three odor treatments, including CO_2 alone or in combination with 1-octen-3-ol and indole. We divided the ROI into three sections (ROI-1,2,3, Fig. 5A) to explore possible differences in terms of trajectory speed, velocity and tortuosity. Kernel density estimations of mosquito locations along the X-axis were statistically different between the three treatments (Fig. 5B). A bird eye view (X-Y plane) of flight trajectories representing flight speed suggested differences between the treatments (Fig. 5C, see example of individual trajectories in Additional file 8: Fig. S3). 1-Octen-3-ol seemed to increase the number of trajectories and coverage of the ROI while indole appeared to reduce flight speed across that same area. Among all three ROIs, speed was higher and more consistent in response to CO_2 in ROI-1-2 than in ROI-3 (Fig. 5D) (Kruskal-Wallis H test, $H = 86.8$, $df = 2$, $P < 0.0001$). As a result, we focused on ROI-1&2 for further analyses. As reflected in Fig. 5C, the addition of 1-octen-3-ol elicited higher speeds than with CO_2 alone (Fig. 5E). The addition of indole elicited significant decreases in speed compared to those observed

with CO₂ alone or in combination with 1-octen-3-ol (Kruskal-Wallis H test, $H = 301.2$, $df = 2$, $P < 0.0001$). While upwind velocity did not show any statistical differences between CO₂ and 1-octen-3-ol, indole elicited lower upwind velocities (Fig. 5F) (Kruskal-Wallis H test, $H = 35.5$, $df = 2$, $P < 0.0001$). Finally, indole-induced tortuosity was significantly higher than those elicited by CO₂ alone or in combination with 1-octen-3-ol (Fig. 5G) (Kruskal-Wallis H test, $H = 20.9$, $df = 2$, $P < 0.0001$).

Discussion

The overlapping chemical structures between DEET and indole provided the initial impetus to test the potential blocking effect of the latter on AaegOR8. We observed a significant reduction in the current baseline of OR8 at high indole concentrations, and indole generated (*R*)-1-octen-3-ol-dependent hyperpolarization currents (Fig. 1C). In comparison to DEET and IR3535, these increases in current baseline were robust and exhibited different degrees of reversibility with indole showing the highest level of insurmountability. This finding indicates that the affinity of indole for OR8 is higher than for the other two tested insect repellents. We also did not observe that indole affected whole cell currents of oocytes expressing Orco, suggesting that indole acts as an allosteric modulator of OR8.

We had previously reported on these currents – elicited by the highest concentrations of DEET, IR3535 and picaridin – without providing a molecular mechanism underlying this phenomenon [4, 6]. Recently, similar observations were observed with *Cx. quinquefasciatus* and *An. gambiae* ORs suggesting that these hyperpolarization currents may be mediated by chloride influx [24, 25]. The reversible decrease of the baseline current in OR8-injected oocyte suggests that these hyperpolarization currents may not be generated by the activation and subsequent opening of the OR8 ion channel but may reflect a reduction of its constitutive activity. This interpretation is consistent with the moderate yet significant increase in baseline current.

What is the ecological role of (*R*)-1-octen-3-ol? *OR8* is expressed in the maxillary palps of adult mosquitoes [10, 11]. It is selectively activated by nanomolar concentrations of (*R*)-1-octen-3-ol when expressed in *Xenopus* oocytes [12]. The evidence presented in this study suggest a correlative relationship between the observed repellency and one possible molecular mechanism for eliciting this behavior. We recognize that indole repellency may be generated by other molecular targets, such as the indolergic or other receptors. Indeed, indole-induced repellency may have no direct relevance to 1-octen-3-ol/OR8-mediated behavior. The high concentrations used here (up to 1 M) are unlikely to be found in nature. However, at the lower used concentrations, we also overserved significant repellency. Whether the same or other detection mechanisms are involved is not known. It is possible that different indole concentrations target different molecular mechanisms as observed with DEET [4, 5, 26–30].

Indole is synthesized by bacteria [31, 32], fungi, plants [33] and released by animals [34]. Indole may attract female mosquitoes in the contexts of animal host- [35] and oviposition-seeking [36]. In *Culex* mosquitoes, indole has been associated with fermented Bermuda grass infusions, which attract gravid

female mosquitoes [37–39]. However, indole alone does not appear to act as an oviposition attractant in *Ae. aegypti* [18] or in *An. gambiae*, while it is slightly repellent to ovipositing *Ae. albopictus* [18].

Indole may play a role in human host-seeking as well [36]. Microbiota on the skin [40] and in sweat [34] release indole and may contribute to its attractiveness. We tested the olfactory-mediated effect of multiple indole doses in the context of human-host seeking and provide observational evidence that indole repels female mosquitoes (Fig. 4). Whether these doses are ecologically relevant is not known but could provide one explanation as to why different individuals exhibit different levels of attractiveness towards mosquitoes.

To test whether indole-mediated OR8 inhibition would elicit repellency, we tested its effect against blood-seeking female *Ae. aegypti*. Our results suggest that indole acts as an olfactory repellent in a broad range of doses (1 M – 10^{-5} M) and loses its repellent activity at low doses (10^{-6} M). We used DEET as a positive control since it is a recognized and effective insect repellent. DEET probably operates through different modes of action, including interacting with both smell and taste receptors [4, 5, 26–30], and by reducing the volatility of odorants [8]. In our arm-in-a-cage assay, indole was not applied to the skin but on a physically separate chemical holder, suggesting that the inhibitory effect of indole is not chemically decreasing odorant volatility ('masking') as it has been suggested in *Anopheles gambiae* [7–9]. The number of mosquito visits was the most significant measure of repellency. By contrast, the durations of the visits were highly variable and did not show any significant differences between treatments. Taken together, these results suggest that mosquitoes are repelled before making contact with the net, which is located immediately around the chemical source. This would be consistent with an olfactory-mediated effect, whereas once landed, olfactory information may be downplayed by the brain while other senses, such as taste or close range chemosensation take precedence.

The highest indole concentrations used in our behavioral experiments are unpleasant (moth ball) to the human nose and may elicit rejection from consumers. However, we have shown that indole concentrations as low as 10^{-5} M elicit significant repellency at short range. These concentrations are consistent with the fragrance industry, which uses indoles in dilutions of 0.1% or less to create a floral effect in perfumes. In addition, indole may be mixed with other volatiles to enhance this floral effect.

Conclusions

Our study provides strong support that indole, a mosquito kairomone of unclear ecological significance, is inhibiting human-host seeking *Ae. aegypti* females. We also provided pharmacological evidence that the OR8/1-octen-3-ol detection pathway is a potential molecular mode of action for this inhibition. Since indole activates multiple mosquito ORs, including OR2 and OR10, the indole-mediated reduction in anemotactic flight may be caused by distinct olfactory pathways. Our observations nevertheless raise the need for additional studies on the efficacy of indole as a potential mosquito spatial repellent.

Declarations

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Figures

Figure 1

Indole inhibits OR8-Orco activation by (*R*)-1-octen-3-ol. A) (*R*)-1-octen-3-ol is an alkenyl alcohol and a potent ligand of OR8. Indole is an aromatic bicyclic organic compound. *N,N*-Diethyl-*meta*-toluamide (DEET) is a benzyl-ester sharing a benzene ring and a nitrogen atom with indole (red features). **B)** Representative current traces of AaegOR8-Orco activation by 10^{-7} M (*R*)-1-octen-3-ol (black arrowheads)

or by a mixture of 10^{-3} M indole (red arrowhead) and 10^{-7} M (*R*)-1-octen-3-ol (black arrowhead). Normalized responses of AegOR8-Orco to 10^{-7} M (*R*)-1-octen-3-ol alone (control) or in combination with 10^{-3} M indole (indole). Statistical significance was determined using the Wilcoxon–Mann–Whitney test ($n = 5-6$). Orco-injected oocytes respond to 10^{-3} M of the Orco agonist VUAA1 but not to 10^{-3} M indole ($n = 4$). **C**) Representative current traces of AegOR8-Orco activation by (*R*)-1-octen-3-ol alone or in the presence of 10^{-4} M, 10^{-3} M or 10^{-2} M indole. Arrowheads above the traces indicate the onset of the odorant stimulus, black arrowheads represent (*R*)-1-octen-3-ol and red arrowheads represent indole. **D**) Concentration-response relationships of AegOR8-Orco in response to increasing indole concentrations. Current amplitudes were normalized to the maximum response. Statistical significance was determined using a One-Way ANOVA ($P = 0.0142$) followed by a Dunn's multiple comparisons test ($n = 4-7$). **E**) Non-normalized concentration-current response relationships ($n = 4-7$). **F**) Histogram of the depolarization current response amplitudes of AegOR8-Orco in response to (*R*)-1-octen-3-ol alone (black) or in combination with various indole concentrations (shades of red). Statistical significance was determined using multiple t-test and statistical significance determined by the Holm-Sidak method (*: $P \leq 0.05$, **: $P \leq 0.01$, ***: $P \leq 0.001$, $n = 3-7$). **G**) Concentration-response relationships of indole-induced hyperpolarization currents exhibited by AegOR8-Orco-injected oocytes.

Figure 2

High indole concentrations elicit both depolarization and hyperpolarization currents. **A)** Representative current traces of AaegOR8-Orco activation by 8 sec stimulations of (*R*)-1-octen-3-ol alone or increasing indole concentrations (10^{-6} - 10^{-2} M). Arrowheads above the traces indicate the onset of the odorant stimulus, black arrowheads represent (*R*)-1-octen-3-ol and red arrowheads represent indole. **B)** Representative current trace of AaegOR8-Orco activation by 10^{-7} M (*R*)-1-octen-3-ol (black arrowhead) and

by 10^{-2} M indole (red arrowhead). Statistical significance was determined using the Wilcoxon–Mann–Whitney test ($n = 7$). **C**) (*R*)-1-Octen-3-ol is required to elicit significant indole-induced hyperpolarization currents. Nanomolar concentrations of (*R*)-1-octen-3-ol induce larger indole-induced depolarization currents than indole alone. Representative traces are shown above. Statistical significance was determined using a t-test ($n = 7$ -12).

Figure 3

Indole and insect repellents increase the baseline current state of AaegOR8-Orco-injected oocytes.

Representative current traces of AaegOR8-Orco successively exposed to 100 nM (*R*)-1-octen-3-ol under ND96 (black trace), continuous perfusion under indole (0.005 M), DEET (0.01 M), and IR3535 (0.01 M) perfusions (red traces) with increasing concentrations of (*R*)-1-octen-3-ol, ND96 perfusion, and 100 nM (*R*)-1-octen-3-ol. The same protocol was applied to water-injected oocytes as well. In AaegOR8-Orco-injected oocytes, perfusion buffer transitions from ND96 to indole, DEET or IR3535 are shown as solid red arrows, while the reverse perfusion transitions are shown as a solid black arrow. In water-injected oocytes, the corresponding buffer transitions are indicated by empty red and black arrows. The baseline currents of OR8-Orco-injected oocytes exhibit significant increases and decreases compared to water-injected oocytes. Statistical significance was determined using the Wilcoxon–Mann–Whitney test ($n = 5$ -9). **B**) Under saline perfusion (ND96), AaegOR8-Orco elicits a stable current baseline. A stimulus pulse of (*R*)-1-octen-3-ol activates AaegOR8-Orco, which translates into a transient whole cell depolarization current. A pulse of (*R*)-1-octen-3-ol (low concentration) and indole (high concentration) mixture transiently induces a hyperpolarization current. Increasing (*R*)-1-octen-3-ol concentrations reverses indole blockade. High indole concentrations elicit small depolarization and hyperpolarization currents. Under indole perfusion, the current baseline is increased.

Figure 4

Indole repels human-seeking female *Ae. aegypti* mosquitoes. **A**) Diagram of the arm-in-cage assay (inset). The gloved experimenter's hand is introduced inside a cage containing 15 *Ae. aegypti* female mosquitoes. The exposed skin area was monitored by a recording camera (see Additional file 6: Video S1 for a representative video). The number of mosquito visits were counted and normalized as a function of treatments, including diethyl ether (vehicle), DEET 1 M and seven indole concentrations. All treatment groups were analyzed using a Wilcoxon rank-sum test ($n = 3$, see methods section for more details). Percentage of inhibition are indicated below the graph in the grey shaded area. **B**) Hyperbola line fitting of the cumulative number of mosquito landings over time. Points represented are mean $\pm$ SEM ($n = 3$). See Additional file 7: Fig. S2. **C**) Histogram of the normalized average time duration mosquitoes spent on the exposed skin area. A one-way ANOVA analysis was used to analyze all treatment groups. Shapiro-

Wilk normality test ($W = 0.95056$, $P = 0.2212$) was performed on the square root of the dependent variable ($n = 3$).

Figure 5

Indole reduces anemotactic behavior induced by CO₂ and 1-octen-3-ol. **A)** Schematic of the flight tunnel. The region of interest (ROI) was equally divided into three sections represented by differentially shaded volumes (1-3). **B)** Kernel density estimations of mosquito coordinates within the first 3 min of odorant-induced flight within the ROI. Statistical significance is in parentheses. **C)** Bird eye view of the collective flight trajectories elicited by the three odor treatments. Each visible dot represents a trajectory coordinate. Speed levels along the trajectory are color-coded according to the speed index (mm/sec). Graded areas below the X-axes represent the three sections of the region of interest. **D)** Violin plot of mosquito speed in ROIs 1, 2 and 3. Median speeds are provided in the table below the plot as well as the statistical

significance. **E**) Speed in ROI (1+2) as a function of the odor treatments. **F**) Upwind velocity in the ROI (1+2) as a function of the odor treatments. **G**) Tortuosity in ROI (1+2) as a function of the odor treatments. The CO₂ treatment is green colored, the CO₂ + 1-octen-3-ol treatment is colored in blue, and the CO₂ + 1-octen-3-ol + indole treatment is colored in maroon. CO₂, 600 ppm, 1-octen-3-ol, 168 mg, indole, 29 mg. ROI, region of interest, Stat. sig., statistical significance.

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