orco mutant mosquitoes lose strong preference for humans and are not repelled by volatile DEET

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Female mosquitoes of some species are generalists and will blood-feed on a variety of vertebrate hosts, whereas others display marked host preference. Anopheles gambiae and Aedes aegypti have feeds on a variety of vertebrate hosts, whereas others display marked host preference. Anopheles gambiae and Aedes aegypti have fed on a variety of vertebrate hosts, whereas others display marked host preference. Anopheles gambiae and Aedes aegypti have fed on a variety of vertebrate hosts, whereas others display marked host preference. Anopheles gambiae and Aedes aegypti have fed on a variety of vertebrate hosts, whereas others display marked host preference. Anopheles gambiae and Aedes aegypti have fed on a variety of vertebrate hosts, whereas others display marked host preference. Anopheles gambiae and Aedes aegypti have fed on a variety of vertebrate hosts, whereas others display marked host preference. Anopheles gambiae and Aedes aegypti have fed on a variety of vertebrate hosts, whereas others display marked host preference. Anopheles gambiae and Aedes aegypti have fed on a variety of vertebrate hosts, whereas others display marked host preference. Anopheles gambiae and Aedes aegypti have fed on a variety of vertebrate hosts, whereas others display marked host preference. Anopheles gambiae and Aedes aegypti have fed on a variety of vertebrate hosts, whereas others display marked host preference. Anopheles gambiae and Aedes aegypti have fed on a variety of vertebrate hosts, whereas others display marked host preference. Anopheles gambiae and Aedes aegypti have fed on a variety of vertebrate hosts, whereas others display marked host preference. Anopheles gambiae and Aedes aegypti have fed on a variety of vertebrate hosts, whereas others display marked host preference. Anopheles gambiae and Aedes aegypti have fed on a variety of vertebrate hosts, whereas others display marked host preference. Anopheles gambiae and Aedes aegypti have fed on a variety of vertebrate hosts, whereas others display marked host preference. Anopheles gambiae and Aedes aegypti have fed on a variety of vertebrate hosts, whereas others display marked host preference. Anopheles gambiae and Aedes aegypti have fed on a variety of vertebrate hosts, whereas others display marked host preference. Anopheles gambiae and Aedes aegypti have fed on a variety of vertebrate hosts, whereas others display marked host preference. Anopheles gambiae and Aedes aegypti have fed on a variety of vertebrate hosts, whereas others display marked host preference. Anopheles gambiae and Aedes aegypti have fed on a variety of vertebrate hosts, whereas others display marked host preference. Anopheles gambiae and Aedes aegypti have fed on a variety of vertebrate hosts, whereas others display marked host preference. Anopheles gambiae and Aedes aegypti have fed on a variety of vertebrate hosts, whereas others display marked host preference.

orco mutant olfactory sensory neurons have greatly reduced spontaneous activity and lack odour-evoked responses. Behaviourally, orco mutant mosquitoes have severely reduced attraction to honey, an odour cue related to floral nectar, and do not respond to human scent in the absence of CO2. However, in the presence of CO2, female orco mutant mosquitoes retain strong attraction to both human and animal hosts, but no longer strongly prefer humans. orco mutant females are attracted to human hosts even in the presence of DEET, but are repelled upon contact, indicating that olfactory- and contact-mediated effects of DEET are mechanistically distinct. We conclude that the odorant receptor pathway is crucial for an anthropophilic vector mosquito to discriminate human from non-human hosts and to be effectively repelled by volatile DEET.

In the vinegar fly, Drosophila melanogaster, Orco is an obligate olfactory co-receptor that forms a complex with all ligand-selective odorant receptors and is required for efficient trafficking to olfactory sensory neurons.

Figure 1 | Targeted mutagenesis of orco in A. aegypti. a, Snake plot of A. aegypti Orco with amino acids colour-coded to indicate conservation with D. melanogaster. The amino acids encoded by the DNA bound by the orco ZFN (blue) and the epitope of the Drosophila anti-Orco antibody (green) are indicated. b, Analysis of orco ZFN mutagenesis in A. aegypti G0 and G1 animals assayed by Illumina sequencing of an amplicon containing the ZFN cut site. c, Frequency of insertions or deletions expressed as the number of sequence reads per million reads. d, Schematic of orco ZFN pair binding to orco DNA (top). orco mutant alleles (bottom). e, f, Immunofluorescence of frozen sections of wild-type (e) and orco mutant (f) antennae (D. melanogaster Orco antibody staining, green; DAPI (4',6-diamidino-2-phenylindole) nuclear stain, blue). Scale bar, 10 μm.
Figure 2 | Reduced spontaneous activity and loss of odour-evoked responses in orco mutant olfactory neurons. a, b, Spontaneous activity in the small-amplitude cell in capitate-peg sensilla on the maxillary palp (a) or in the large-amplitude cell in short blunt-tipped sensilla on the antenna (b) recorded from the indicated genotypes. Data are plotted as mean ± s.e.m. (maxillary palp n = 19 (+/+), n = 5 (orco<sup>16/+</sup>), n = 21 (orco<sup>16</sup>), n = 25 (orco<sup>5</sup>), n = 15 (orco<sup>5/16</sup>); antenna n = 20 (+/+), n = 20 (orco<sup>16/+</sup>), n = 20 (orco<sup>5/16</sup>)). Values that are significantly different are labelled with different letters (one-way ANOVA for both comparisons, P < 0.0001 followed by Tukey’s HSD test). c, Representative spike traces of maxillary palp neurons stimulated with (R)-1-octen-3-ol (10<sup>–6</sup> dilution in paraffin oil) for 1 s (red bar). Small-amplitude spikes are in red. d, Summary of small-amplitude spikes evoked by (R)-1-octen-3-ol (solid bars) or paraffin oil solvent (open bars). Data are presented as mean ± s.e.m. e, Representative spike traces of maxillary palp neurons stimulated with CO<sub>2</sub> (0.05%) for 1 s (grey bar). Large amplitude spikes are in grey. f, Summary of large-amplitude spikes evoked by CO<sub>2</sub> (solid bars) or air (open bars). Data are plotted as mean ± s.e.m. (n = 7 (+/+/), n = 6 (orco<sup>16/+</sup>), n = 10 (orco<sup>5/+</sup>), n = 9 (orco<sup>16</sup>), n = 8 (orco<sup>5</sup>), n = 6 (orco<sup>5/16</sup>)). There was no difference in CO<sub>2</sub>-evoked spikes between genotypes except for those between orco<sup>16/</sup> and orco<sup>5/16</sup> (one-way ANOVA, P = 0.0233). g, Representative spike traces of short blunt-tipped antennal neurons of the indicated genotypes stimulated for 1 s (black bar) with 10% ethyl butyrate. h, Odour-evoked responses of 20 short blunt-tipped sensilla for each of the indicated genotypes.

Neuron dendrites<sup>4</sup>. We reasoned that mutations in the orco gene of <i>A. aegypti</i> should eliminate signalling mediated by all 131 ligand-selective ORs in this mosquito<sup>5</sup>. To obtain <i>A. aegypti</i> heritable targeted null mutations in the orco gene, we used zinc-finger nucleases (ZFNs), which are fusion proteins of a sequence-specific DNA binding protein and a nuclelease that induces mutagenic double-stranded breaks<sup>6,9,11</sup>.

We first used ZFNs<sup>12</sup> to disrupt green fluorescent protein (GFP) in a transgenic strain of <i>A. aegypti</i> and detected a wide range of insertion and deletion events in GFP (Supplementary Fig. 1). We next injected ZFNs designed to target the orco locus (Fig. 1a) into wild-type mosquito embryos. The orco ZFNs induced mutations at high efficiency in adults derived from injected embryos (G<sub>0</sub>) as well as their progeny (G<sub>1</sub>) (Fig. 1b–d). Three mutant alleles were characterized, orco<sup>2</sup>, orco<sup>5</sup> and orco<sup>16</sup>, and these are predicted to produce truncated Orco protein. We did not detect Orco expression in antennae in any of these homoygous orco mutant alleles (Fig. 1e, f and data not shown). Using these homoygous mutants, we further generated heterolelic orco mutant combinations to control for independent background mutations.

orco mutants exhibited severely impaired electrophysiological responses (Fig. 2). Spontaneous activity in maxillary palp (Fig. 2a) and antennal (Fig. 2b) sensilla was reduced relative to wild-type and heterozygous controls, as observed previously in <i>D. melanogaster</i> orco mutants<sup>2</sup>. We were unable to locate any maxillary palp capitate-peg sensilla responsive to a ligand for OR8/Orco, (R)-1-octen-3-ol<sup>13–15</sup>, in orco<sup>5</sup>, orco<sup>16</sup> or orco<sup>5/16</sup> mutant mosquitoes (0/25, 0/21 and 0/15 sensilla tested, respectively), but readily detected them in wild-type and heterozygous orco<sup>16/+</sup> animals (19/29 and 5/7 sensilla tested, respectively) (Fig. 2c, d). Insect chemosensory responses to carbon dioxide (CO<sub>2</sub>) are orco-independent<sup>6</sup> and capitate-peg sensilla in all genotypes responded normally to CO<sub>2</sub> (Fig. 2e, f). On the antennae of orco mutants, we were unable to locate any short blunt-tipped trichoid sensilla responding to a panel of odors selected from those previously shown to activate neurons in subsets of these sensilla<sup>17</sup> (Fig. 2g, h). As expected, a subset of short blunt-tipped sensilla from wild-type (13/20) and heterozygous (13/20) control mosquitoes responded to one or more odors in the panel (Fig. 2g, h). Our results show that in orco mutants, both antennal and maxillary palp neurons fail to respond to the odours tested, suggesting that as in <i>D. melanogaster</i>, orco is required for the function of ligand-selective ORs.

We investigated whether these electrophysiological defects translate into altered responses to important olfactory cues using a modified two-port Gouck olfactometer<sup>18</sup> (Fig. 3a). Both male and female mosquitoes feed on nectar to satisfy their metabolic needs and are attracted to floral and honey odours<sup>19</sup>. We quantified attraction of fasted male mosquitoes into altered responses to important olfactory cues using a modified two-port Gouck olfactometer<sup>18</sup> (Fig. 3a). Both male and female mosquitoes feed on nectar to satisfy their metabolic needs and are attracted to floral and honey odours<sup>19</sup>. We quantified attraction of fasted male mosquitoes feed on nectar to satisfy their metabolic needs and are attracted to floral and honey odours<sup>19</sup>.
female mosquitoes did not respond to unworn sleeves, but showed moderate attraction to human-scented sleeves (Fig. 4b). In contrast, orco mutant females showed little or no response to host odour (Fig. 4b). Because live hosts emit both odour and CO₂, we repeated these experiments in the presence of CO₂ (Fig. 4c). Wild-type female mosquitoes showed moderate attraction to CO₂ alone, and this response was greatly enhanced when human-scented sleeves were presented along with CO₂ (Fig. 4c). Contrary to the defect seen in the absence of CO₂, orco mutant females exhibited the same robust response to human odour in the presence of CO₂ as controls (Fig. 4c). Likewise, orco mutant females responded at the same robust levels as controls to air that had passed over a live human arm, supplemented with human breath, and tested in a modified version of the same olfactometer (Fig. 4a, d).

These results indicate that CO₂ synergizes with host odour to rescue the defect in orco mutant attraction, suggesting that mosquitoes possess redundant mechanisms for host odour detection that can only be activated in the presence of CO₂. Further, this indicates that olfactory signalling mediated through a second family of chemosensory receptors, the ionotropic receptors, cannot compensate for the loss of OR function in orco mutants in the absence of CO₂.

Most populations of A. aegypti strongly prefer human over non-human hosts, prompting us to ask whether the OR pathway is required for host discrimination. We assayed host discrimination between human and guinea-pig (Cavia porcellus)—an animal host to which A. aegypti has been shown to respond 46. Both wild-type and orco mutants showed moderate levels of baseline attraction to a live guinea-pig (Fig. 4e). We were intrigued by the stronger attraction of orco mutants to guinea-pig relative to controls, but note that this difference was statistically significant for only orco5/16. We then offered mosquitoes a choice between air that had passed over a live guinea-pig or the arm of one of 14 live human subjects (Fig. 4f). orco mutants were less likely than wild-type to choose the human host, resulting in a statistically significant lower mean preference index (Fig. 4f). To determine if the reduced preference of mutant mosquitoes for humans depended on host-specific odours alone, we repeated the choice assay but replaced live hosts with host odour collected on nylon sleeves, supplemented with CO₂ (Fig. 4g). Under these conditions, control

Figure 3 | Disruption of honey odour detection in orco mutants. a, Diagram of two-port olfactometer used for honey response assay. b, c, Response of male (b) or female (c) mosquitoes to honey (beehive) or glycerol by the indicated genotypes. Wild-type males distributed evenly between the two ports when both contained honey (Student’s t-test, \( P = 0.13; n = 8 \)), and did not respond to glycerol \( (n = 7) \). Genotypes varied significantly in their response to honey (one-way ANOVA, \( P < 0.001; n \) (males) = 10–11; \( n \) (females) = 7–11). Genotypes marked with different letters are significantly different by post hoc Tukey’s HSD test. When fasted, we found no reduction in survival or locomotor activity in orco mutants relative to controls (Supplementary Fig. 2). Thus, these weak responses cannot be explained by a locomotion defect in the mutants.

Female mosquitoes additionally feed on vertebrate hosts to acquire nutrients necessary for egg development and are attracted to host odour. We examined responses of female mosquitoes to human odour trapped on nylon sleeves using the same two-port olfactometer, modified to include carbon-filtered air flow (Fig. 4a). In this assay, wild-type

Figure 4 | Disruption of host detection and discrimination in orco mutants. a, Diagram of modifications to the two-port olfactometer for host assays with female mosquitoes. b, Response to no stimulus (open box), unworn nylon sleeve (sleeve cartoon), or human scented nylon sleeve (dotted hand cartoon) without CO₂. Genotypes varied significantly (one-way ANOVA, \( P < 0.0001; n = 15 \)). c, Response to CO₂ or human scented nylon sleeve with CO₂. Genotypes did not differ in their response to human scented nylon sleeves with CO₂ (one-way ANOVA, \( P = 0.8881; n = 15 \)). d, Response to no stimulus, human breath (face cartoon), or live human (blue). Genotypes did not differ in response to human scented nylon sleeves with CO₂ (one-way ANOVA, \( P = 0.8881; n = 15 \)). e, Response to no stimulus or guinea-pig (red). Genotypes differed in responses (one-way ANOVA, \( P = 0.0001; n = 12–13 \)). f, Preference index for live human or guinea-pig. Variation was significant among both genotypes and human subjects tested (two-way ANOVA, \( P < 0.0001 \) for genotype and \( P = 0.0015 \) for subject). g, Preference index for human or guinea-pig-scented nylon sleeve. Variation among genotypes was significant (one-way ANOVA; \( P < 0.0001; n = 5–6 \)).
mosquitoes strongly preferred human odour, whereas orco mutants again showed only a weak preference (Fig. 4g). Overall response rates did not differ between genotypes in either assay (Supplementary Fig. 3a–d). We speculate that orco mutants are attracted to vertebrate hosts, but are impaired in their ability to discriminate between them.

ORs have been proposed to be the major target of the olfactory effect of the insect repellent DEET\(^{20–25}\). To investigate whether DEET can interfere with the strong attraction to human hosts in orco mutants, we tested female orco mutant and control mosquitoes in a two-port olfactometer DEET choice assay (Fig. 5a). Wild-type and heterozygous mosquitoes avoided the port with 10% DEET and accumulated in the solvent port (Fig. 5a). In contrast, orco mutants were insensitive to DEET and accumulated equally in both ports. Differences in the overall response rate were not statistically significant (Supplementary Fig. 3c, f).

The DEET choice assay tests mosquito response over a distance of 20 to 100 cm. To test the olfactory effect of DEET at close range, we used a host proximity assay that measures the effects of DEET over a distance of 2.5 to 32.5 cm (Fig. 5b). In this assay, a human arm treated with solvent or 10% DEET was placed 2.5 cm from the side of a screened cage. Control mosquitoes were attracted to the arm when it was treated with solvent, but strongly avoided it when it was treated with DEET. In contrast, orco mutants showed equal attraction to the solvent- and DEET-treated arm (Fig. 5b and Supplementary Video 1). These results indicate that the olfactory attraction of orco mutants for humans cannot be inhibited by DEET, even at close range.

Upon landing on a host, mosquitoes are exposed to taste cues and become susceptible to the contact-mediated repellent effects of DEET. In this study, we investigated the requirement for orco and the OR pathway in mosquito appetitive behaviour and sensitivity to the insect repellent DEET. orco mutants are impaired in both honey and host odour attraction. Despite this defect in host odour detection, orco mutants can still respond to live hosts. We propose that CO\(_2\), along with odours that are detected by the ionotropic receptor pathway, may constitute more generic vertebrate signals that are sufficient to drive attraction.

The impairment in host preference in orco mutant females suggests a specialization of the OR pathway in A. aegypti for host odour discrimination. We speculate that the OR/Orco pathway provides information about the specific identity of the host and that specific ORs may mediate preference for humans in this anthropophilic disease vector. A role for ORs in host discrimination is consistent with the broader range of ligands that activates this class of receptors compared to ionotropic receptors.

Our results also provide proof that orco and the OR pathway are necessary for the olfactory effects of DEET on mosquitoes. A previous study used RNA interference to knock down orco in A. gambiae larvae\(^{23}\) and found that these animals no longer responded to DEET. However, because mosquito larvae are aquatic, these experiments could not distinguish olfactory- and contact-mediated effects. Three mechanisms have been suggested to explain the olfactory repellency of DEET, but no clear consensus has emerged. First, DEET may silence ORs tuned to attractive odours\(^{20,21}\). Second, DEET may activate one or a few ORs to trigger repulsion\(^{22,23}\). Third, DEET may act as a ‘confusant’ to modulate the activity of many ORs\(^{24,25}\). The fact that orco
mutants retain very strong olfactory host attraction indicates that the first hypothesis cannot be correct. If the olfactory repellency of DEET acted solely to inhibit the OR pathway, DEET would not be an effective insect repellent. Instead, our results are consistent with the second or third hypotheses. Our genetic analysis of orco mutants indicates that either mechanism must overcome the strong baseline attraction to hosts in the presence of CO₂ seen in orco mutants. Finally, we note that the development of an efficient method to generate targeted mutations in A. aegypti opens up this important disease vector to comprehensive genetic analysis.

METHODS SUMMARY

orco ZFNs were generated using the CompoZr Custom ZFN Service (Sigma-Aldrich). Of the 16 ZFN pairs screened, the orco exon 1 ZFN pair used in this study had the greatest activity, comparable to a highly active positive control ZFN that targets CCR5. Mutant alleles were detected by Illumina sequencing of an study had the greatest activity, comparable to a highly active positive control ZFN pair that targets CCR5. Mutant alleles were detected by Illumina sequencing of an amplicon that contained the ZFN cut site. Mutant alleles were isolated using size-based genotyping of amplicons surrounding the deletion site, allowing us to discriminate heterozygous from homozygous individuals. Mosquitoes were tested for their response to odour cues in a modified version of a Gouck two-port olfactometer. Full Methods are described in the Supplementary Information.

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Supplementary Information is available in the online version of the paper.

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Author Contributions M.D. carried out the experiments in Fig. 1 and Supplementary Fig. 1 and Supplementary Fig. 2b. C. E.J.D. carried out the experiments in Supplementary Fig. 2a. N.J. generated the GFP transgenic A. aegypti and injected the GFP ZFN for Supplementary Fig. 1 and was supervised by A.A.J. T.N. carried out the experiments in Fig. 2. G.G. reared mosquitoes and genotyped orco mutants. C.S.M. developed the assays used in Figs 3 and 4 with M.D. and L.S. M.D. and C.S.M. carried out the experiments in Figs 3 and 4. L.S., E.J.D. and M.D. developed and carried out the assays in Fig 5. M.D., C.S.M. and L.B.V. wrote the paper.

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