

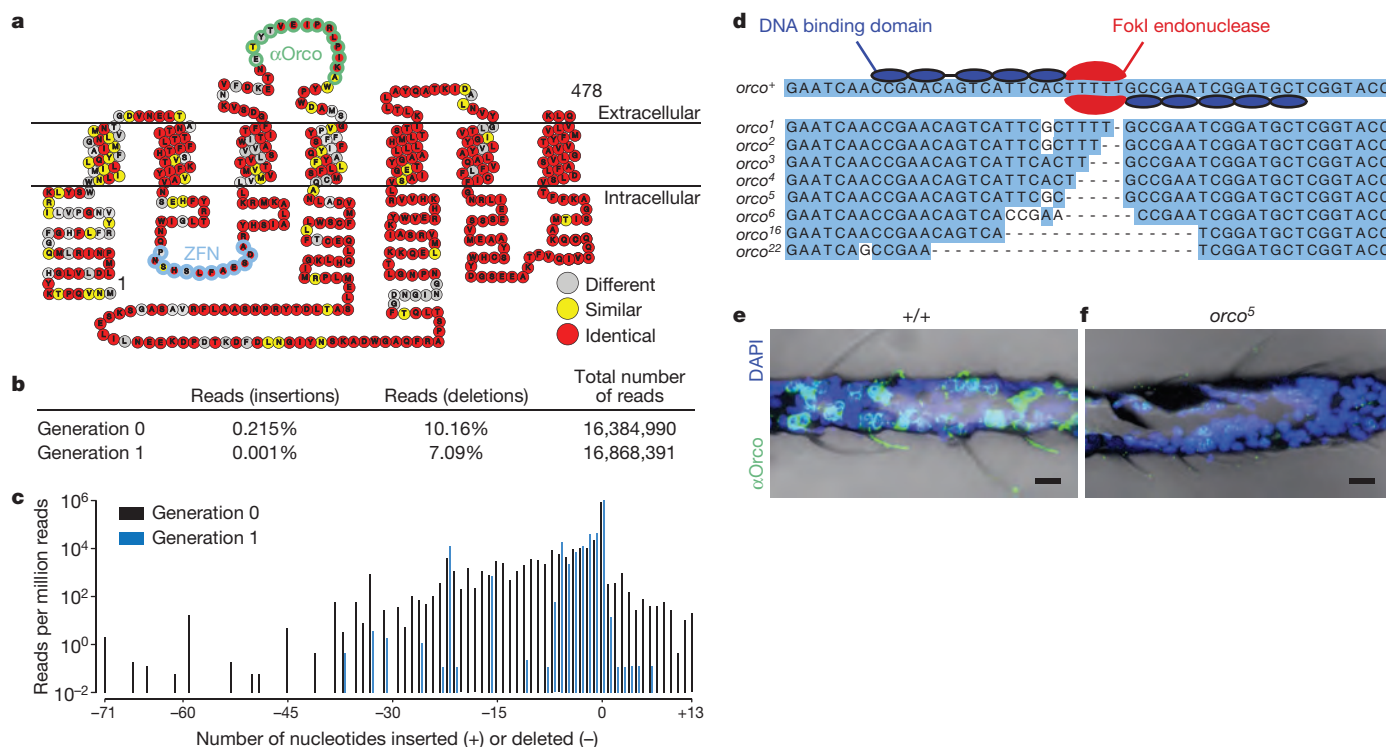
# orco mutant mosquitoes lose strong preference for humans and are not repelled by volatile DEET

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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 evolved a strong preference for humans, making them dangerously efficient vectors of malaria and Dengue haemorrhagic fever<sup>1</sup>. Specific host odours probably drive this strong preference because other attractive cues, including body heat and exhaled carbon dioxide (CO<sub>2</sub>), are common to all warm-blooded hosts<sup>2,3</sup>. Insects sense odours via several chemosensory receptor families, including the odorant receptors (ORs), membrane proteins that form heteromeric odour-gated ion channels<sup>4,5</sup> comprising a variable ligand-selective subunit and an obligate co-receptor called Orco (ref. 6). Here we use zinc-finger nucleases to generate targeted mutations in the *orco* gene of *A. aegypti* to examine the contribution of Orco and the odorant receptor pathway to mosquito host selection and sensitivity to the insect repellent DEET (*N,N*-diethyl-*meta*-toluamide).

*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 CO<sub>2</sub>. However, in the presence of CO<sub>2</sub>, 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.

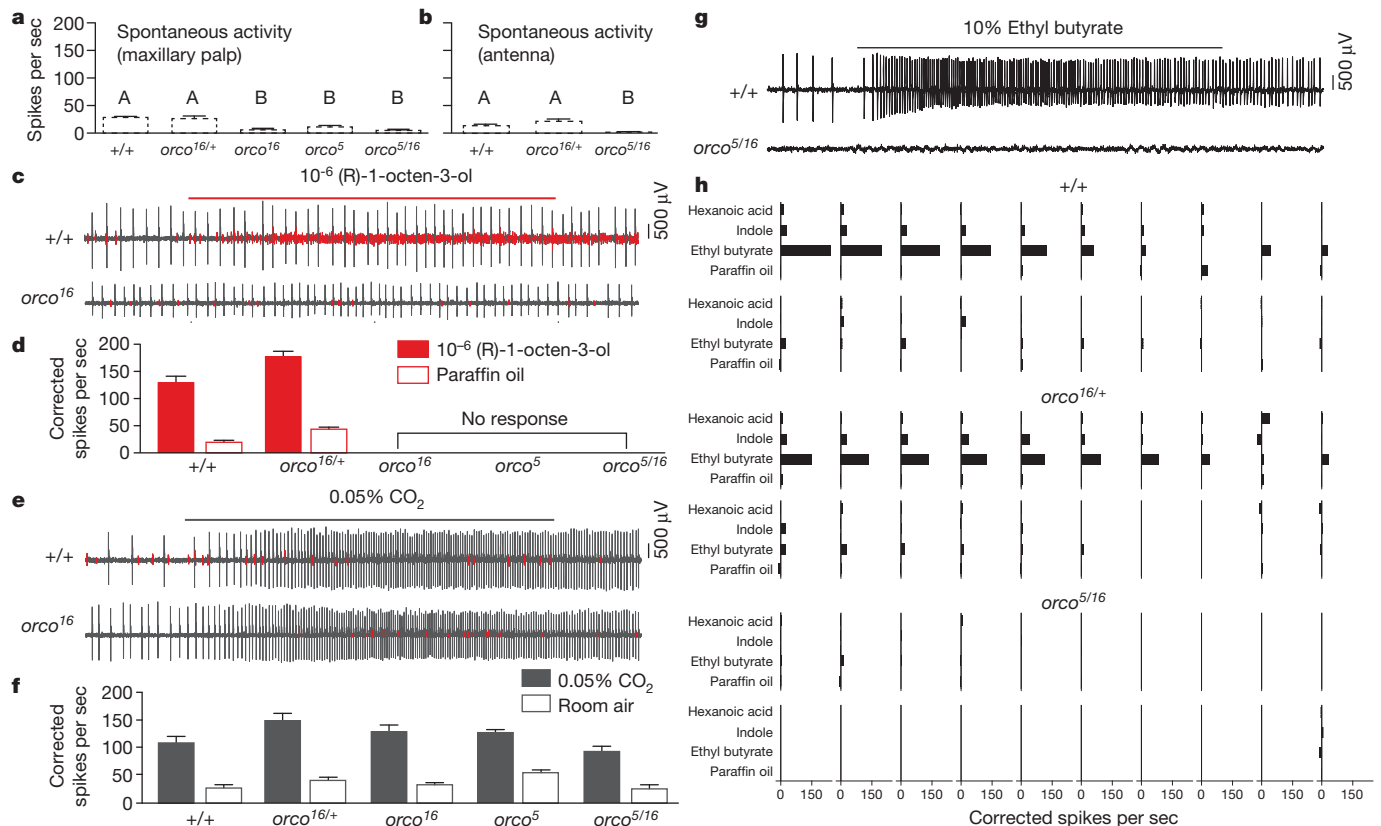
In the vinegar fly, *Drosophila melanogaster*, Orco is an obligate olfactory co-receptor<sup>7</sup> that forms a complex with all ligand-selective odorant receptors and is required for efficient trafficking to olfactory sensory



**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* G<sub>0</sub> and G<sub>1</sub> 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*<sup>5</sup> mutant (**f**) antennae (*D. melanogaster* Orco antibody staining, green; DAPI (4',6-diamidino-2-phenylindole) nuclear stain, blue). Scale bar, 10 μm.

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**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  $\pm$  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 ( $10^{-6}$  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-

neuron dendrites<sup>8</sup>. We reasoned that mutations in the *orco* gene of *A. aegypti* should eliminate signalling mediated by all 131 ligand-selective ORs in this mosquito<sup>9</sup>. To obtain *A. aegypti* 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 nuclease that induces mutagenic double-stranded breaks<sup>10,11</sup>.

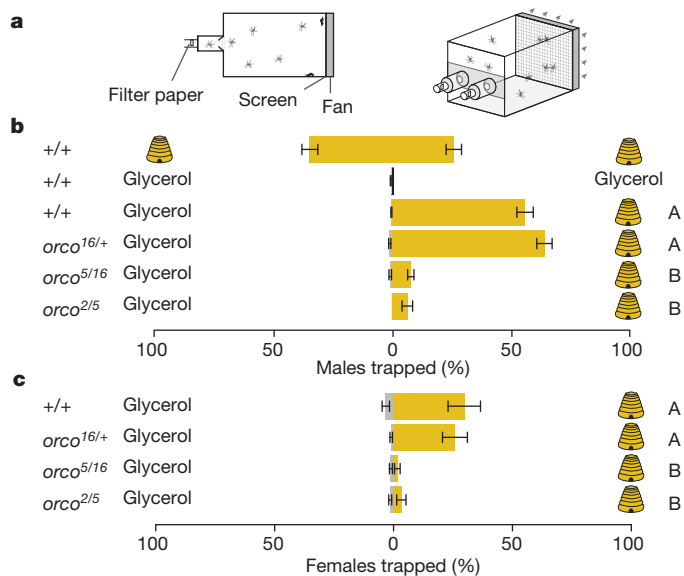
We first used ZFNs<sup>12</sup> to disrupt green fluorescent protein (GFP) in a transgenic strain of *A. aegypti* and detected a wide range of insertion and deletion events in GFP (Supplementary Fig. 1). We next injected ZFNs designed to target the *orco* locus (Fig. 1a) into wild-type mosquito embryos. The *orco* ZFNs induced mutations at high efficiency in adults derived from injected embryos ( $G_0$ ) as well as their progeny ( $G_1$ ) (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 homozygous *orco* mutant alleles (Fig. 1e, f and data not shown). Using these homozygous mutants, we further generated heteroallelic *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 *D. melanogaster orco* mutants<sup>7</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

3-ol (solid bars) or paraffin oil solvent (open bars). Data are presented as mean  $\pm$  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  $\pm$  s.e.m. ( $n = 7$  (+/+),  $n = 6$  (*orco*<sup>16/+</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,  $P = 0.0232$ ). **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.

*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>16</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 odors tested, suggesting that as in *D. melanogaster*, *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 odors<sup>19</sup>. We quantified attraction of fasted male and female mosquitoes to honey or glycerol, which is odourless and has a viscosity and water content similar to honey. Although wild-type and heterozygous control mosquitoes responded to honey, heteroallelic *orco* mutant mosquitoes showed little to no response (Fig. 3b, c).



**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.

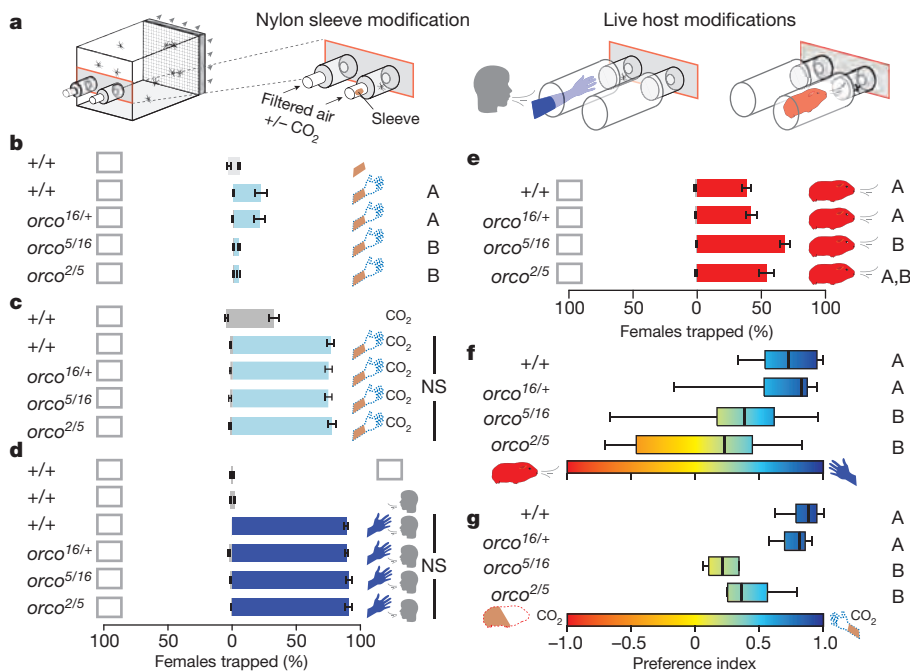
When fasted, we found no reduction in survival or locomotor activity in *orco* mutants relative to controls (Supplementary Fig. 2). Thus, these weak responses cannot be explained by a locomotion defect in the mutants.

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

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 CO<sub>2</sub>, we repeated these experiments in the presence of CO<sub>2</sub> (Fig. 4c). Wild-type female mosquitoes showed moderate attraction to CO<sub>2</sub> alone, and this response was greatly enhanced when human-scented sleeves were presented along with CO<sub>2</sub> (Fig. 4c). Contrary to the defect seen in the absence of CO<sub>2</sub>, *orco* mutant females exhibited the same robust response to human odour in the presence of CO<sub>2</sub> 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 CO<sub>2</sub> 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 CO<sub>2</sub>. 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 CO<sub>2</sub>.

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<sup>18</sup>. 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 *orco*<sup>5/16</sup>. 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 CO<sub>2</sub> (Fig. 4g). Under these conditions, control



**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 CO<sub>2</sub>. Genotypes varied significantly (one-way ANOVA,  $P < 0.0001$ ;  $n = 15$ ). **c**, Response to CO<sub>2</sub> or human scented nylon sleeve with CO<sub>2</sub>. Genotypes did not differ in their response to human scented nylon sleeves with CO<sub>2</sub> (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 (one-way ANOVA,  $P = 0.8$ ;  $n = 7$ ). **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;  $P < 0.0001$ ;  $n = 5-6$ ). Data in **b-e** are plotted as mean  $\pm$  s.e.m. and data in **f, g** are presented as box plots (black line, median; bounds of boxes, first and third quartiles; bars, range). In **b** and **e-g**, genotypes marked with different letters are significantly different by post hoc Tukey's HSD test. NS, not significant.



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<sup>20–25</sup>. 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. 3e, f).

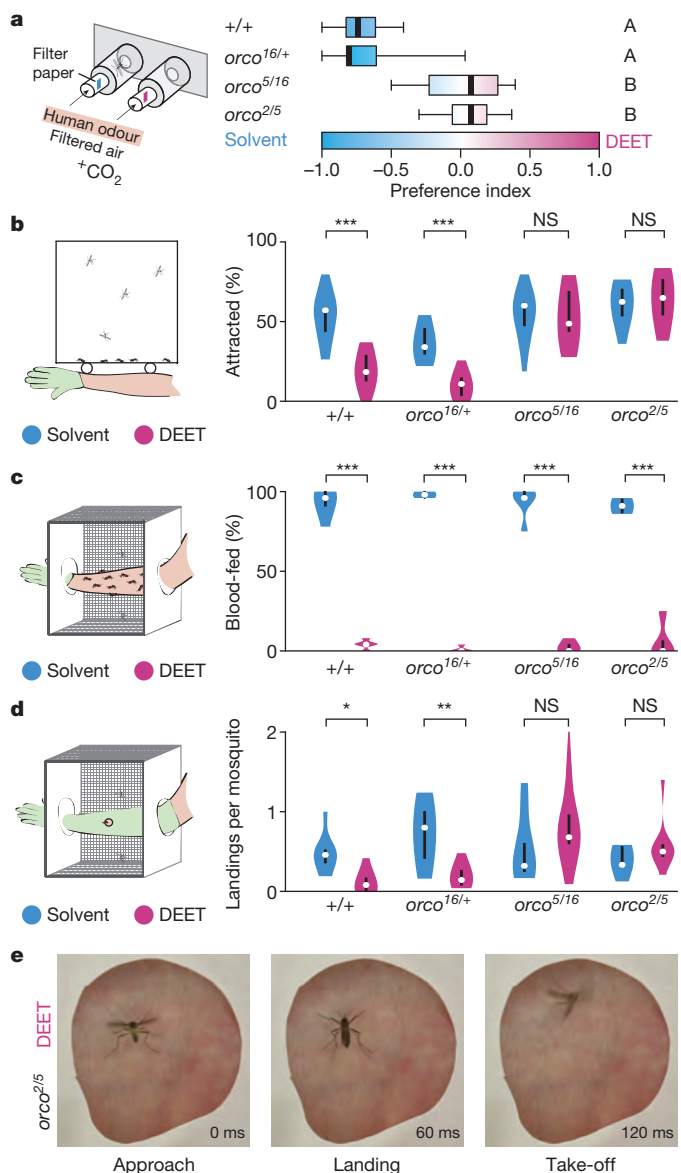
The DEET choice assay tests mosquito response over a distance of 20 to 100 cm. To test the olfactory effect of DEET at close range, we used a host proximity assay that measures the effects of DEET over a distance of 2.5 to 32.5 cm (Fig. 5b). In this assay, a human arm treated with solvent or 10% DEET 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. Work in *D. melanogaster* indicates that DEET acts on gustatory receptors to induce taste avoidance<sup>26</sup>. To investigate whether *orco* mutants are sensitive to the contact-mediated effects of DEET, we carried out an arm-in-cage biting assay in which subjects inserted a solvent- or DEET-treated arm into a mosquito cage (Fig. 5c). DEET treatment inhibited blood feeding of both wild-type and *orco* mutant females (Fig. 5c). To detect *orco* mutants contacting DEET-treated skin, we designed a landing assay to image female mosquitoes interacting with exposed skin treated with DEET or solvent (Fig. 5d). In contrast to controls, *orco* mutants approached the host and briefly contacted the DEET-treated skin as often as solvent-treated skin (Fig. 5d). A representative *orco*<sup>2/5</sup> mutant mosquito is repelled within 60 msec of contact with DEET-treated skin (Fig. 5e). These results support the conclusion that although the olfactory effect of DEET requires an intact OR pathway, the contact repellent activity of DEET is independent of *orco* function.

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<sub>2</sub>, 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<sup>27,28</sup> compared to ionotropic receptors<sup>29</sup>.

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<sup>23</sup> 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



**Figure 5 | Female *orco* mutants are insensitive to volatile DEET but are repelled on contact.** **a**, Preference for human host-scented traps containing either solvent (cyan) or 10% DEET (magenta). Data are presented as box plots (black line, median; bounds of boxes, first and third quartiles; bars, range). Genotypes marked with different letters are significantly different by post hoc Tukey's HSD test (one-way ANOVA;  $P < 0.0001$ ;  $n = 7-16$ ). *orco* mutant preference is not significantly different from zero (one sample Student's *t*-test; *orco*<sup>2/5</sup>,  $P = 0.3$ ; *orco*<sup>5/16</sup>,  $P = 0.9$ ). **b**, Per cent mosquitoes attracted to a human arm treated either with solvent or 10% DEET (Bonferroni corrected *t*-test comparing solvent and DEET; \*\*\* $P < 0.00025$ ; NS, not significant;  $n = 12-14$ ). **c**, Per cent females blood-fed on a human arm treated with solvent or 10% DEET (Bonferroni corrected *t*-test comparing solvent and DEET; \*\*\* $P < 0.00025$ ;  $n = 4-5$ ). **d**, Per cent landing on human skin treated either with solvent or 10% DEET (Bonferroni corrected *t*-test comparing solvent and DEET; \* $P < 0.0125$ , \*\* $P < 0.0025$ ;  $n = 9$ ). The violin plots in **b–d** show the median as a white dot and the first and third quartile by the bounds of the black bar. The violins are clipped at the range of the data, except in **d**, in which the *orco*<sup>5/16</sup> DEET violin is further clipped at 2.0, excluding a single outlier at 3.76. **e**, Video stills separated by 60 msec of an *orco*<sup>2/5</sup> mutant mosquito responding to DEET-treated skin.

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<sup>20,21</sup>. Second, DEET may activate one or a few ORs to trigger repulsion<sup>22,23</sup>. Third, DEET may act as a 'confusant' to modulate the activity of many ORs<sup>24,25</sup>. 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 CO<sub>2</sub> 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 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<sup>18</sup>. Full Methods are described in the Supplementary Information.

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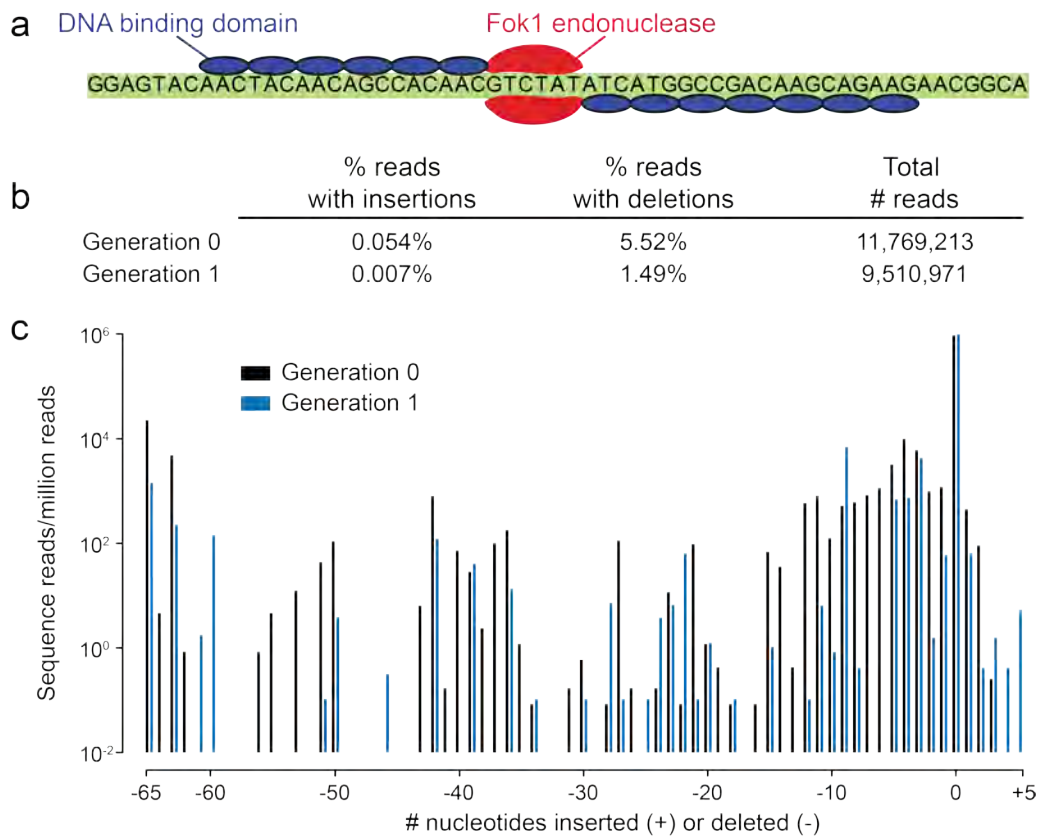
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Supplementary Information is available in the online version of the paper.

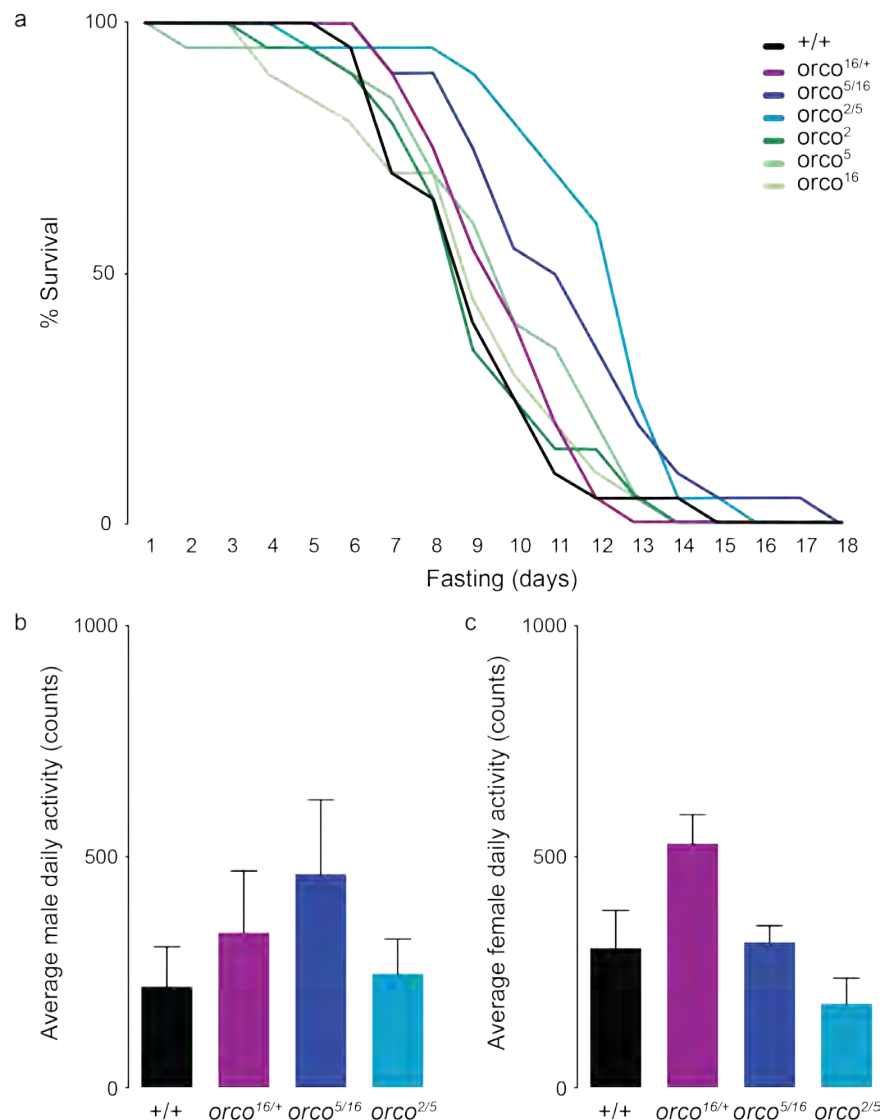
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**Author Contributions** M.D. carried out the experiments in Fig. 1 and Supplementary Fig. 1 and Supplementary Fig. 2b, c. E.J.D. carried out the experiments in Supplementary Fig. 2a. N.J. generated the GFP transgenic *A. aegypti* and injected the GFP ZFN for Supplementary Fig. 1 and was supervised by A.A.J. T.N. carried out the experiments in Fig. 2. C.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. 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.

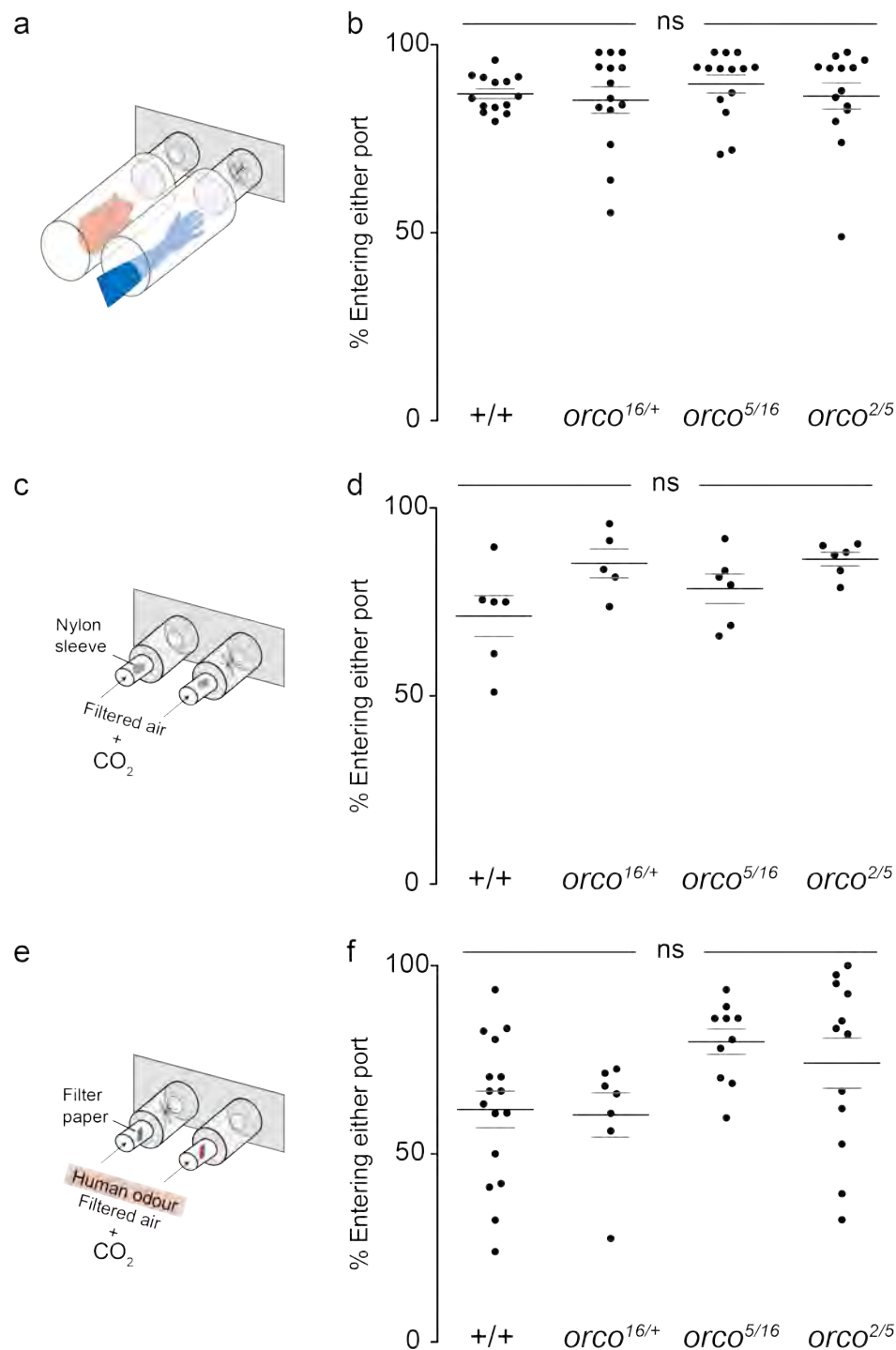
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**Supplementary Figure 1 | Targeted mutagenesis of GFP with zinc-finger nucleases in transgenic *A. aegypti*.** **a**, Top: schematic of ZFN pair binding to GFP DNA sequence. **b**, Analysis of GFP ZFN mutagenesis in G<sub>0</sub> and G<sub>1</sub> *Aedes aegypti* assayed by Illumina sequencing of an amplicon containing the ZFN cut site. **c**, Frequency of insertions/deletions expressed as the number of sequence reads per million reads.



**Supplementary Figure 2 | Fitness and locomotor activity of *orco* mutants when fasted. a,** Survival of genotypes under fasting. Per cent daily survival of 20 individuals from each genotype (10 males, 10 females) is plotted. Significance was determined using log rank test and Geahand-Wilcoxon test followed by pairwise log rank comparisons with Bonferroni correction (corrected significance threshold;  $p < 0.0024$ ). Using this test,  $orco^{2/5}$  mosquitoes lived significantly longer than wild-type,  $orco^2$ ,  $orco^{16}$ , and  $orco^{16/+}$  mosquitoes. There was no difference for any other pair of curves. **b** and **c**, Average daily locomotor activity of males (**b**) and females (**c**) after 4 days of fasting measured by number of infrared beam breaks (counts). There was no statistical difference between genotypes in the male assay (1-way ANOVA,  $p = 0.4797$ ). In females, the only statistically significant difference was between  $orco^{16/+}$  and  $orco^{2/5}$  (1-way ANOVA,  $p = 0.0053$  followed by Tukey's HSD test).



**Supplementary Figure 3 | Overall response rates in 2-port olfactometer choice assays. a-c,** Percentage of female mosquitoes entering either port in **a**, live host preference assay (see **Fig. 4f**; 1-way ANOVA,  $p=0.7412$ ;  $N=14$ ), **b**, host preference assay with odour trapped on nylon sleeves (see **Fig. 4g**; 1-way ANOVA,  $p=0.0514$ ;  $N=5-6$ ), or **c**, two-port olfactometer assay with DEET versus solvent (see **Fig. 5a**; 1-way ANOVA,  $p=0.0550$ ;  $N=7-16$ ). No significant difference in overall response was found in any assay.



## Supplementary methods

**Insect rearing.** Mosquitoes (*Aedes aegypti*) were maintained at 25–28°C with 70–80% relative humidity under a 14 hr light:10 hr dark cycle (lights on 8 am). The Orlando strain was used as wild type. Eggs were hatched in deoxygenated, deionized water containing powdered Tetramin tropical fish food (Tetra, Melle, Germany). Larvae were cultured in deionized water and fed Tetramin tablets. Adults were given unlimited access to 10% sucrose solution. Adult females were blood-fed on mice to generate eggs for the behaviour experiments and on an arm of a human volunteer to isolate mutant alleles. All blood-feeding procedures with mice and humans were approved and monitored by The Rockefeller University Institutional Animal Care and Use Committee (IACUC) and Institutional Review Board (IRB), respectively. All human subjects gave their informed consent to participate in these experiments.

**Zinc-finger nuclease (ZFN) reagents.** The zinc-finger pair that targets GFP has been used previously to disrupt GFP<sup>1</sup> and was provided by Sigma-Aldrich Life Science (St. Louis, MO). The DNA sequence bound by the left and right GFP ZFNs is indicated by the dark blue ovals on Supplementary Figure 1a. David Briner at Sigma-Aldrich Life Science provided project support for *orco* ZFN production using the CompoZr<sup>®</sup> Custom ZFN technology. The DNA sequence bound by the left and right *orco* ZFNs is indicated by the dark blue ovals on Figure 1d. The 5 base-pair (bp) gap in length between ZFNs permits wild-type FokI endonuclease dimerization and DNA cleavage.

***Aedes* transgenic line and ZFN mutagenesis.** GFP ZFNs were injected into embryos of a transgenic *A. aegypti* line that expresses GFP in the eye and which was produced by standard microinjection techniques using *Mos1/mariner* transposon vectors<sup>2</sup>. The transgene contains the 3xP3 eye promoter<sup>3</sup> driving eGFP expression. GFP-positive individuals were crossed to Higgs mosquitoes and screened by Southern blot to identify single insertions. The ZFN pair against GFP was successfully used in the rat<sup>1</sup>.

ZFN-injected  $G_0$  embryos were raised to adulthood and crossed together to generate generation one ( $G_1$ ) progeny. To detect mutations in the GFP locus caused by ZFN activity, we amplified a 112 bp fragment that encompasses the ZFN binding sites. Both forward and reverse primers contain both gene-specific sequences (bold) and those necessary to prime Illumina sequencing and together these produced a 204 bp amplicon:

Forward: 5' AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACG  
ACGCTCTTCCGATCT**CAAGGAGGACGGCAACATCCTGGGGC**3'

Reverse: 5' CAAGCAGAAGACGGCATACGAGCTCTTCCGATCT**GATCTTGAAGTTCAC**  
**CTTGATGC**3'

Genomic DNA was prepared from 135  $G_0$  males in 6 independent preparations of ~22 mosquitoes each and 1800  $G_1$  mosquitoes in 36 independent preparations of ~50 mosquitoes each. Each of the 6  $G_0$  and 36  $G_1$  genomic preparations was used as a template for a separate PCR reaction performed with Accuprime high fidelity PCR mix (Invitrogen, Carlsbad, CA). The 6 independent  $G_0$  PCRs were pooled and Illumina sequenced (76 bp reads, single end) generating 12,206,513 reads. All Illumina sequencing was carried out at The Rockefeller University Genomics Resource Center on a Genome Analyser IIx instrument (Illumina, San Diego, CA) using standard sample preparation and loading protocols obtained from the manufacturer. The 36 independent  $G_1$  PCRs were similarly pooled and sequenced (80 bp reads, single end) yielding 10,727,425 reads. Reads were subjected to quality filtering [90% of bases with a q-score (phred) greater than 25]. We also required that the reads contain the forward primer sequence (CAAGGAGGACGGCAACATCCTGGGGC) with one mismatch allowed. Primer filtering generated 11,979,134  $G_0$  reads and 10,433,144  $G_1$  reads. 636,325 reads that contained TGATAC in  $G_1$  sequencing were a result of a PCR contaminant and were removed from the analysis. Reads were trimmed using clipping adapter sequences (GCCGAC & GCATCA) and their sizes compared. The amplicon contained 65 bp encompassing the ZFN binding site, where sequence

changes could be detected. All trimming, quality filtering, and selection of primer containing reads were done using components of the FASTX Toolkit (Hannon Laboratory; [http://hannonlab.cshl.edu/fastx\\_toolkit/index.html](http://hannonlab.cshl.edu/fastx_toolkit/index.html)). We detected a wide range of insertion and deletion events in both  $G_0$  and  $G_1$  samples, suggesting that ZFN-mediated mutations are efficiently generated and transmitted to the germ line in mosquitoes (Supplementary Fig. 1b-c).

***orco* mutant allele generation and detection.** Genetic Services Inc. (Cambridge, MA, USA) injected the *orco* ZFN mRNAs into ~1000 *A. aegypti* Orlando strain embryos at a concentration of 400 ng/ $\mu$ l using embryo preparation methods described previously<sup>4,5</sup>.  $G_0$  males and females were sexed during pupation, allowed to mate freely after they were sexually mature, and females were blood-fed to generate  $G_1$  progeny. The 76 gravid  $G_0$  females were put into oviposition vials in groups of 5 to collect  $G_1$  eggs. Genomic DNA was extracted from the 71  $G_0$  males and mutations were detected using Illumina sequencing as described for the Supplementary Figure 1b-c. Primers were designed to amplify a 135 bp region encompassing the *orco* ZFN target site. Primers contain both gene specific sequences (bold) and sequences necessary for Illumina genotyping and produce a 227 bp amplicon:

Forward: 5' AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCT  
TCCGATCTCCGCACGCTGGGCATCTGGAATC3'

Reverse: 5' CAAGCAGAAGACGGCATAACGAGCTCTTCCGATCTACGGATAGCACTGTA  
GTCACCAT3'

The amplicon was amplified with Accuprime high-fidelity PCR mix and Illumina sequenced (76 bp, single end) yielding 16,837,291 quality filtered reads [90% of bases with a q-score (phred) greater than 25]. Reads that contained the locus-specific forward primer sequence with up to one mismatch were used in the analysis (16,400,396 reads). To determine their size, reads were trimmed using clipping adapter sequences (CCGAAT, CCATTC, ACTACA, &

CATGGT). The amplicon included 96 bp encompassing the ZFN binding site, where sequence changes could be detected.

To isolate mutant alleles,  $G_1$  males and females that eclosed in same-sex cages were grouped in polygamous families of 3 males and 3 females. The  $G_1$  polygamous families were allowed to mate and were blood-fed to generate  $G_2$  progeny. Genomic DNA was extracted from  $G_1$  parents. Transmission of mutant alleles was detected using 76 bp Illumina sequencing exactly as described for  $G_0$  parents except that we used bar-coded versions of the forward primer (Bar-coded forward primer 5' AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGAC GCTCTTCCGATCTxxxxCCGCACGCTGGGCATCTGGAATC 3'). The barcodes (xxxx) used were actg, agtc, ctga, cgta, gcta, tagc, tgac, gtca, ctag, gtag, atgt, agtg, actc, agag, and acac.

Genomic DNA from fifteen  $G_1$  families was pooled and PCR was performed using Accuprime high fidelity PCR mix with a bar-coded forward primer. Two lanes of Illumina sequencing (76 bp reads, single end) were performed yielding 17,442,194 quality filtered reads, which were mapped to specific families using the barcode information. To determine the number of insertions/deletions, reads that contained the locus-specific forward primer sequence with up to one mismatch were used (17,126,419 reads). To determine their size, reads were trimmed using clipping adapter sequences (CCGAAT, ACCATT, and CGGTACC). All trimming, quality filtering, selection of primer-containing reads, and bar code filtering was carried out using components of the FASTX Toolkit.

**Establishment of mutant lines** Eggs from families in which *orco*<sup>2</sup>, *orco*<sup>5</sup>, and *orco*<sup>16</sup> alleles were detected were hatched, and pupae were sexed and allowed to eclose in same-sex cages. For *orco*<sup>2</sup> and *orco*<sup>5</sup> alleles, virgin  $G_2$  females were allowed to mate freely with introduced wild-type males 24 hr after eclosion. For *orco*<sup>16</sup>, single pair crosses of  $G_2$  siblings were set up. Females were blood-fed and placed in individual oviposition vials. After eggs were laid, females were



sacrificed and genomic DNA was isolated. A fluorescent amplicon (140 bp in wild-type, 138 bp in *orco*<sup>2</sup>, 135 bp in *orco*<sup>5</sup>, and 124 bp in *orco*<sup>16</sup>) was generated using Accuprime PCR mix (Invitrogen) for each female using the following primers:

Forward: 5' 6-FAM fluorescent modification-TAGCTCCGCACGCTGGGCATCTGGAATC 3'

Reverse: 5' ACGGATAGCACTGTAGTCACCAT 3'

Amplicons were analysed by capillary gel electrophoresis using an Applied Biosystems 3730 DNA analyser. Subsequent generations of mosquitoes were intercrossed and genotyped until homozygous mutant lines were established.

The *orco* mutant alleles were named for the number of deleted bases. These alleles are predicted to produce truncated Orco proteins that delete more than 70 per cent of the wild-type protein (478 amino acids). The frameshift in *orco*<sup>2</sup> alters the peptide sequence after amino acid residue 114 to CRIGCSVPFDCVG and leads to a premature stop after residue 127. The frameshift in *orco*<sup>5</sup> alters the peptide sequence after residue 113 to RRIGCSVPFDCVG, leading to a stop after residue 126. The frameshift in *orco*<sup>16</sup> alters the peptide sequence after residue 112 to RMLGTIRLRWLRCENCWSW, resulting in a stop after residue 131. Homozygous mutant mosquitoes have no obvious defects in viability, fecundity, or external morphology.

**Immunostaining.** Mosquito heads were dissected into 4% paraformaldehyde in PBT [phosphate-buffered saline (PBS) plus 0.1% Triton X-100] on wet ice and fixed for 30 min at 4°C. After three 5 min washes with PBS, the fixed heads were cryoprotected with 25% sucrose in PBT overnight at 4°C. Heads were frozen in Tissue-Tek OCT compound (Sakura Finetek, Alphen aan den Rijn, The Netherlands) for 20 min on dry ice, cryosectioned at a thickness of 10–15 µm, and mounted on Superfrost plus slides (Fisher Scientific, Pittsburgh, PA). Tissue sections were dried for 30 min at room temperature and post-fixed with 4% paraformaldehyde in PBT at room temperature for 30 min. The slides were washed 3 times for 5 min in PBT, blocked in 5%

normal goat serum in PBT for 1 hr at room temperature, and incubated with the *Drosophila* anti-Orco EC2 antibody (1:500 dilution; antigen indicated on Fig. 1a)<sup>6</sup> in 5% normal goat serum in PBT overnight at 4°C. After washing 3 times for 5 min in PBT, the tissue sections were incubated with Alexa Fluor 488 goat anti-rabbit IgG (1:500 dilution; Catalogue #A-11008; Invitrogen) in 5% normal goat serum in PBT at room temperature for 3 hr. After three 5 min washes with PBT, the slides were mounted in Vectashield with DAPI to visualize nuclei (Catalogue #H-1200; Vector Labs, Burlingame, CA, USA). Samples were imaged on an LSM 510 laser scanning confocal microscope (Carl Zeiss Microimaging, Thornwood, NY, USA) at The Rockefeller University Bio-imaging Resource Center. Images shown are maximum projections of 5 sections from a 20 µm image stack.

**Single sensillum electrophysiology.** Extracellular recordings of mosquito olfactory sensilla were carried out as described<sup>7</sup>. Female mosquitoes (*Aedes aegypti*) were recorded at 7-14 days after adult eclosion between 11am and 5pm, corresponding to ZT3 to ZT9. Olfactory sensilla on the antenna and maxillary palp were subjected to extracellular recording using a 10x AC probe connected to an IDAC4 amplifier (Syntech, Kirchzarten, Germany). The reference electrode was placed into the eye. The recording electrode was inserted into the peg-shaped capitate-peg sensillum on the maxillary palp or short blunt-tipped sensilla on the antenna. Spontaneous and odour-evoked extracellular spikes were recorded<sup>8</sup>. Two to three sensilla were sampled from each animal mounted for recording.

Nearly 100% of these capitate-peg sensilla gave strong responses to CO<sub>2</sub> as shown by an increase in frequency of action potentials from a large-amplitude spiking neuron<sup>9</sup>. A subset of these CO<sub>2</sub>-sensitive sensilla also contained a smaller amplitude cell sensitive to (R)-1-octen-3-ol, which is alternately referred to as the “B” cell<sup>10</sup> or “C” cell<sup>9</sup>. To avoid confusion with the names of the (R)-1-octen-3-ol cell, we refer to the CO<sub>2</sub> cells as large-amplitude spiking and the (R)-1-octen-3-ol as small-amplitude spiking neurons.

We recorded from 60 short blunt-tipped sensilla in segments 6 to 10 of the antenna. Sensilla were chosen only by their morphology as no odorant receptor-based odour response map of the *Aedes* antenna exists. Each sensillum was tested with a previously defined panel of odours that can evoke activity in subsets of these sensilla<sup>11</sup> consisting of ethyl butyrate (10%), indole (1%), and hexanoic acid (10%) diluted in paraffin oil along with paraffin oil alone. These synthetic odorants were chosen solely because they have been previously shown to activate *Aedes aegypti* olfactory neurons. We make no claims about the behavioural relevance of these odorants or that they are natural ligands that these insects would encounter in the wild.

The majority of wild-type (13/20) and heterozygous *orco*<sup>16</sup> (13/20) sensilla, showed large amplitude “A” cell responses to one or more odours. Smaller amplitude “B” cell spikes were infrequent and found only in wild-type (1/20) and heterozygous *orco*<sup>16</sup> (1/20) sensilla. A sensillum was defined as sensitive to an odour if its response was greater than or equal to 15 corrected spikes/sec.

(R)-1-octen-3-ol (C.A.S. 3687-48-7) was obtained from Bedoukian (Danbury, CT, USA). Ethyl butyrate (C.A.S. 105-54-4), indole (C.A.S. 120-72-9), and hexanoic acid (C.A.S. 142-62-1) were obtained from Sigma-Aldrich. Serial 10-fold dilutions (volume:volume; v:v) in paraffin oil (Sigma-Aldrich) were made as indicated. For indole, a 10% weight:volume stock solution was made in ethanol before dilution in paraffin oil (v:v). 15  $\mu$ L of odorant dilution was applied onto a 3 x 40 mm strip of chromatography filter paper (catalogue #05-714-1; Fisher Scientific) and inserted into a Pasteur glass pipette connected to the Syntech CS-55 stimulus device (Syntech). Odorants were applied to mosquito sensilla for 1 sec using the CS-55 stimulus device, which injected odour stimuli into continuous charcoal-filtered air aimed at the sensilla. CO<sub>2</sub> was purchased from Matheson Tri-Gas (Gloucester, MA) as a custom formulated 10% CO<sub>2</sub>/90% air mixture. For CO<sub>2</sub> stimuli, 10% CO<sub>2</sub> was filled into a 20 mL syringe and mixed with air to prepare the desired concentration (v:v).

Each sensillum was tested only once with a series of stimuli, and consecutive stimuli were applied with an inter-stimulus interval of at least 60 sec. Data were collected using Autospike (Syntech), and exported as ASCII format, and analysed with SpikeClouds, a custom program written by Mathias Ditzen in IDL (ITT Visual Information Solutions, Boulder, CO)<sup>12</sup>. Corrected spike increases were computed by subtracting the average spontaneous activity in 1 sec before odorant stimulus from the average activity during the 1 sec stimulus. For spontaneous activity analysis of capitae-peg sensilla (Fig. 2a), small-amplitude spike number was counted for 1 sec prior to the application of paraffin oil. The same sensilla analysed in Figure 2a were also used to calculate odour-evoked responses to (R)-1-octen-3-ol (Fig. 2d). Spontaneous activity of antennal short blunt-tipped sensilla (Fig. 2b) was determined by counting large-amplitude spikes for 1 sec prior to the application of paraffin oil using the same sensilla as in Figure 2h.

For maxillary palp recordings, each sensillum was challenged first with CO<sub>2</sub> (0.05%). If a CO<sub>2</sub> response was obtained, we proceeded to stimulate the same sensillum with (R)-1-octen-3-ol. Due to inherent variability in recordings or biological variability of the mosquito, not all preparations that gave a response to CO<sub>2</sub> also responded to (R)-1-octen-3-ol. Analysis in Figure 2d was performed only on sensilla that responded to (R)-1-octen-3-ol.

Dose-response curves of wild-type capitae-peg sensilla with (R)-1-octen-3-ol and CO<sub>2</sub> were generated to determine the concentration that produced half-maximal stimulation (EC<sub>50</sub>). (R)-1-octen-3-ol dilutions between 10<sup>-4</sup> and 10<sup>-10</sup> were tested. Wild-type maxillary palp capitae-peg sensilla exhibited small-amplitude spikes in response to (R)-1-octen-3-ol with a half-maximal effective dose (EC<sub>50</sub>) of 262 nM (data not shown), which is in the same range as the EC<sub>50</sub> of 158 nM measured for the *A. aegypti* OR8/Orco receptor presumed to be expressed in this maxillary palp neuron<sup>8, 9, 13</sup>. CO<sub>2</sub> responsiveness was tested at concentrations of 0.005%, 0.01%, 0.05%, 0.1%, 0.5% and 1% and the EC 50 was determined to be 0.11%.



**Fasting resistance assay.** Ten male and ten female mosquitoes from each of seven genotypes (*orco*<sup>2</sup>, *orco*<sup>5</sup>, *orco*<sup>16</sup>, *orco*<sup>16/+</sup>, *orco*<sup>5/16</sup>, *orco*<sup>2/5</sup> and wild-type) were tested for fasting resistance. Each individual mosquito was aspirated into a fly vial (25 mm diameter, 95 mm long) containing two cotton balls soaked with 10 mL of distilled water and plugged with a cellulose acetate fly vial plug (Cat. # 49-101, Genesee Scientific, San Diego, CA, USA). Vials were randomized for genotype and gender in the racks, which were then placed in plastic storage bins, covered with black cloth to ensure even dim lighting conditions for all vials, and racks rotated each day to control for any positional effects. Experiments took place in an environmental room set to 25°C and 70-80% relative humidity and a 14 hr light: 10 hr dark cycle, although the black cloth greatly diminished illumination of the animals during the light phase. To quantify fasting resistance, vials were visually examined each day for movement. If no movement visible, the vial was tapped twice and inspected visually. If the mosquito still did not move, it was recorded as dead.

**Locomotor activity assays.** Mosquito activity during fasting was monitored using LAM25 Locomotor Activity Monitors (Trikinetics Inc., Waltham, MA, USA). 5 to 6 day-old sugar-fed male and female mosquitoes were individually placed in glass tubes (25 mm diameter, 125 mm long) under cold anaesthesia. A water-soaked cotton plug sealed one end of the glass tube and served as a water source for the mosquito. The vials were placed in the monitoring device inside a Digitherm incubator (Tritech Research Inc., Los Angeles, CA, USA) set to 25°C and 70-80% relative humidity under a 14 hr light: 10 hr dark cycle. Infrared beam breaks triggered by the mosquito's movement were recorded every second and tabulated into 1 min bins. Bins with 60 or more beam breaks and trials with 2000 or more beam breaks per day were excluded from the analysis. For Supplementary Figure 2 b-c, activity was calculated for 24 hr on the fourth full day of fasting.

**Two-port olfactometer assays.** The olfactometer, modified from one originally described by Gouck<sup>14</sup>, consisted of a large plexiglass box (50 x 50 x 80 cm) connected to two smaller cylindrical traps at one end (18 cm long, 9 cm inner diameter), which were in turn connected to

two stimulus ports (12.7 cm long and 7.5 cm inner diameter for the honey/nylon sleeve and DEET assays in Fig. 3, 4b, 4c, 4g, 5a, or 38 cm long and 13.65 cm inner diameter for the live-host assay in Fig. 4d-f). The opposite end of the main compartment was covered with a screen and a spongy mesh filter, and then connected to a box fan. When on, the box fan pulled air through the stimulus ports where it mixed with the stimuli, then through the traps and into the main compartment. The rate of air flow in the stimulus ports was 81-84 feet/min as measured by a Velocicalc Air Velocity Meter Model 9515 (T.S.I. Inc., Shoreview, MN USA). A white cloth covered the main compartment to shield external visual cues.

For each trial, 50 adult *A. aegypti* mosquitoes (male or female; 1-3 weeks post eclosion) were sorted under cold anaesthesia (4°C), and placed in a small cage. All females used in these assays were mated previously but had not taken a blood meal. Females were fasted with no access to water for 16-24 hr prior to host odour and DEET assays. Before testing in the honey assay, male and female mosquitoes were fasted for 4 days with access to water. Pre-assay fasting and behaviour experiments took place at 25°C and 70-80% relative humidity. In our experiments, we tested four genotypes: wild-type (+/+), heterozygous (*orco*<sup>16/+</sup>), and heteroallelic *orco* mutants (*orco*<sup>5/16</sup> and *orco*<sup>2/5</sup>). Heteroallelic mutants were tested to control for background mutations that may have arisen independently in each strain.

Ten min prior to the start of the assay, mosquitoes were released into the main compartment to acclimate. At the end of the acclimation period, the fan was turned on and a sliding door between the trapping chambers and main compartment was opened to allow air to flow through the apparatus. In the live host and honey assays, ambient air was pulled through the apparatus only by the fan. In the nylon sleeve and DEET assays, carbon-filtered air was pulled simultaneously by the fan and pushed by three pumps (Cole-Parmer Quiet Pressure Pump, Catalogue #79610-81) through a plastic bag connected to the stimulus ports at a rate of 81-84 feet/min. The bag was made of clear 1.1 mm thick, linear low density polyethylene (cat #47732-

706, VWR International, Radnor, PA, USA). The bag was resized to 109 cm x 75 cm using a heat sealer and attached to the three air inlet tubes at one end and the two stimulus ports at the other end to form airtight seals. The number of mosquitoes in each trap was counted after 8 min. The 8 min time point was determined empirically to produce consistently high responses. Stimuli were alternated daily between the left and right ports to control for side bias.

Honey assay: Either 100-150 mg of leatherwood honey (Tasmanian Honey Company, Tasmania, Australia) or glycerol (Catalogue # G5516; Sigma-Aldrich) was applied to each of two 55 mm diameter Whatman filter paper circles (GE Healthcare, Buckinghamshire, UK) 30 min prior to the trial. Circles were hung from the top of the two stimulus ports so that the flat axis of each circle was parallel to airflow.

Live host assay: We assessed the response of female mosquitoes to individual human and guinea pig hosts using a live host assay. All behavioural experiments with guinea pigs and human volunteers were approved by The Rockefeller University IACUC or IRB, respectively. All human subjects gave their informed consent to participate in these experiments. The comfort of the guinea pig was assured by rest and feeding periods outside of the olfactometer between trials and the minimization of any stress while in the olfactometer. Neither the guinea pigs nor human volunteers were bitten by mosquitoes in the course of these assays.

We first examined wild-type response to two blank ports or one blank port vs. human breath, exhaled lightly through a nasal mask and redirected by tubing into the opening of the port once every 30 sec. For human trials, a volunteer inserted his or her forearm up to the elbow (N=7 subjects, 3 female, aged 22-35). The human exhaled lightly through the nose for two seconds into the opening of the human port once every 30 sec to provide a source of CO<sub>2</sub>. For the guinea pig trials, an adult female guinea pig was placed into the port on a soft diaper and its natural breath provided a source of CO<sub>2</sub>. In the host preference assays in Figure 4f, 14 human subjects participated (8 female, aged 22-60). Guinea pig respiration provided the sole source of CO<sub>2</sub> in

the guinea pig port. The human arm port was not supplemented with CO<sub>2</sub>. Preference was quantified using a preference index equal to the number of mosquitoes entering the human trap minus those that entered the guinea pig trap divided by the total number of mosquitoes entering either trap.

Nylon sleeve assays: Nylon sleeves 35cm in length were prepared by removing the toe section of women's sheer knee-high stockings (Duane Reade, New York, NY, USA). Five human volunteers (3 females aged 23-36 and 2 males aged 34-37) wore the nylon sleeves on the arm from the armpit to above the wrist for 24 hr, during which time the volunteers engaged in normal daily activities, including at least 30 min of exercise, but did not shower, wear deodorant, perfume, or scented products. In Figure 4b-c, each genotype was tested 3 times with sleeves from each of the 5 volunteers (N=15 for both experiments). For the nylon sleeve host-preference assay (Fig. 4g), human-scented sleeves were conditioned by a single human volunteer as described above. Guinea pig-scented sleeves were worn by an adult female guinea pig, bunched up on the torso between the front and hind legs, for 24 hr under normal housing conditions. Both human and guinea pig sleeves were stored for up to a month in a -20°C freezer. A new set of sleeves was used on each of two days of testing.

When used in the assay, CO<sub>2</sub> was generated by releasing 10% CO<sub>2</sub> into the port. CO<sub>2</sub> was purchased from GTS-Welco (Allentown, PA, USA) as a custom-formulated 10% CO<sub>2</sub>/90% air mixture. Mixed with the air flowing through the apparatus, the approximate final concentration of CO<sub>2</sub> in the port was 0.2-0.3%, as measured with a CO<sub>2</sub> meter (CARBOCAP® Hand-Held Carbon Dioxide Meter GM70, Vaisala Inc., Woburn, MA, USA). Preference index was quantified as described for the live host assay.

DEET assay: Carbon-filtered air was passed over a single human-scented nylon sleeve and this stream was split between both ports of the olfactometer and supplemented with CO<sub>2</sub> (final concentration 0.2-0.3%) so both ports would be equally attractive to the mosquitoes (Fig. 5a).



Ten min prior to the start of each trial, 100  $\mu$ l of 10% DEET/90% ethanol (DEET) or 100% ethanol (solvent) was applied to a 2 cm x 6 cm Whatman filter paper. One treated filter paper (DEET or solvent) was suspended from the ceiling of each of the stimulus ports so that the flat axis of each was parallel to the airflow. Repellency was quantified using a preference index equal to the number of mosquitoes entering the DEET trap minus those that entered the solvent trap divided by the total number of mosquitoes entering either trap. When both traps contained a filter paper treated with solvent, there was no evidence of side bias for either genotype tested (one sample Student's *t*-test; *orco*<sup>16/+</sup>: *p*=0.1031, *orco*<sup>5/16</sup>: *p*=0.2612).

**Human host proximity assay.** This assay is similar to one described in Stanczyk et al.<sup>15</sup>. For each trial, 25 adult female *A. aegypti* mosquitoes (female; 1-3 weeks post eclosion, mated, but not blood-fed) were sorted under cold anaesthesia (4°C), placed in a small cage, and fasted with no access to water for 16-24 hr prior to assaying. Pre-assay fasting and behaviour experiments took place at 25°C and 70-80% relative humidity. Ten to thirty min prior to the start of the assay, mosquitoes were released into each cage to acclimate. Human volunteers (N=4, 2 females, aged 23-36) applied either 900  $\mu$ l 100% ethanol or 900  $\mu$ l 10% DEET in ethanol to their forearm. A human arm was placed 2.5 cm from one side of a standard 28 x 28 x 28 cm cage (Bioquip, Rancho Dominguez, CA). Mosquitoes could not directly contact the human arm in this assay. To control the distance from the arm to the cage, two clear polystyrene fly food vials (25 mm diameter) were placed flush against the cage and the arm was pressed against the vials. The arm was elevated 2.7 cm by placing it on a plastic microcentrifuge test-tube rack. A fly pad (8.1 x 11.6cm; catalogue #59-114; Flystuff.com, San Diego, CA) was placed on top of the cage to diffuse 10% CO<sub>2</sub> into the assay. A Firefly MV camera (Point Grey Research, Richmond, BC, Canada) was positioned to take images of mosquitoes responding to the human arm. The cage and the arm were illuminated by a light box (36.6 cm L x 27.2 cm W x 1.2 cm D; model number #VP-4050L, Smith-Victor Corporation, Bartlett, IL). Trials ran for 4 min and images were acquired at a rate of 1 frame per 5 sec. To quantify mosquito responses, we counted the number

of mosquitoes resting on the lower portion of the screen closest to the human arm, in an area of analysis 25.3 cm wide by 19.3 cm high. We scored the average number of mosquitoes in the analysis area on the last 10 images of each trial divided by the total number of mosquitoes in the assay multiplied by 100, excluding any mosquitoes in flight. Images were cropped to the same size with Fiji software (<http://fiji.sc>) and scored manually with the multipoint counting tool in Fiji.

**Mosquito arm-in-cage biting assays.** Standard assays were carried out<sup>16, 17</sup>, with the modification that the arm of the human subject was kept in the cage for a full five minutes and the total number of females mosquitoes that blood-fed over this interval was scored. For each trial, 25 adult female *A. aegypti* mosquitoes (female; 1-3 weeks post eclosion, mated, but not blood-fed) were sorted under cold anaesthesia (4°C), placed in a small cage, and fasted with no access to water for 16-24 hr prior to assaying. Pre-assay fasting and behaviour experiments took place at 25°C and 70-80% relative humidity. Five to ten min prior to the start of the assay, mosquitoes were released into a standard cage to acclimate. The cages were modified to have two circular openings with cotton sleeves on opposite sides. Human volunteers (N=5, 2 female, aged 23-46) applied solvent or 10% DEET to their forearm exactly as described for the human host proximity assay described above. A subject donned latex gloves to protect the hand and wrist and inserted each arm into a separate cage, such that the gloved hand protruded out of the back of the cage and the elbow rested on the front of the cage. Cotton sleeves were secured against the arm and gloved hand with rubber bands so that mosquitoes could only bite areas of skin that had been treated with DEET or solvent. No synthetic CO<sub>2</sub> was added to these assays, but the tests were carried out in close proximity to the naturally breathing subject. In each trial, one cage contained either wild-type or heterozygous mosquitoes and the second cage contained heteroallelic mutant mosquitoes. Each arm remained in the assay for 5 min, after which the mosquitoes were aspirated into holding chambers, and visually inspected for distended abdomens containing a blood meal under cold anaesthesia (4°C). Mosquitoes that were not visibly blood-

fed were placed between two white paper tissues and gently flattened to release the contents of the gut. Females that produced yellow-clear liquid were scored as not blood-fed and females that produced any amount of blood were scored as blood-fed.

**Mosquito landing assay.** Standard assays were carried out<sup>16, 17</sup> with the modification that the arm of the human subject was kept in the cage for ten minutes and the total number and time of landings and total number of blood-feeding events was scored for each ten minute video. For each trial, 25 adult female *A. aegypti* mosquitoes (female; 1-3 weeks post eclosion, mated, but not blood-fed) were sorted under cold anaesthesia (4°C), placed in a small cage, and fasted with no access to water for 16-24 hr prior to assaying. Pre-assay fasting and behaviour experiments took place at 25°C and 70-80% relative humidity. No synthetic CO<sub>2</sub> was added to these assays, but the tests were carried out in close proximity to the naturally breathing subject. Five min prior to the start of the assay, mosquitoes were released into a standard cage to acclimate. The cages were built with three circular openings with cotton sleeves. One cotton sleeve was fastened around a video camera (Canon EOS-60D with a Canon EF 100mm 1:2.8 macro lens). A human volunteer (one female, age 23 and two males, ages 22 and 37) applied ethanol solvent or 10% DEET in ethanol to the forearm as described for the human host proximity assay above, except 500 µL per trial was applied locally to either the upper half or lower half of the forearm. The subject then put on an elbow-length latex glove with a 2.5 cm diameter circle cut out of it. The arm was then inserted, so the hand rested on the back of the cage and the elbow rested on the front of the cage. Cotton sleeves were secured against the arm with rubber bands so mosquitoes could only land on the glove or skin treated with DEET or solvent. Four trials of solvent or DEET were carried out on a given day, one trial per genotype, with the order randomized. DEET treatments were not mixed with solvent within a given day to avoid possible effects of DEET on solvent controls. To quantify mosquito responses, videos were visually scored for the number of mosquitoes landing on the 2.5 cm circle of exposed skin over a 10 min period, noting the time of each landing, and whether the mosquitoes blood-fed. In some cases the initial landing was on the

latex glove just outside the exposed skin and mosquito walked onto the patch of skin. The total number of mosquitoes in the assay was then divided by the number of landings to derive landings/mosquito. Blood feeding was quantified as the number of mosquitoes that took a complete blood meal divided by the total number of mosquitoes in the assay (solvent: +/+ 34%; *orco*<sup>16/+</sup> 49%; *orco*<sup>5/16</sup> 40%; *orco*<sup>2/5</sup> 32%. DEET: +/+ 0%; *orco*<sup>16/+</sup> 0%; *orco*<sup>5/16</sup> 0%; *orco*<sup>2/5</sup> 0.4%). We assume that blood-feeding rates are lower in this assay than in Figure 5c because a much smaller area of skin is exposed and available for blood-feeding.

**Statistical analysis.** Statistical significance of genotypic differences in spontaneous activity or CO<sub>2</sub> electrophysiological responses were assessed using one-way ANOVA followed by post hoc Tukey's HSD tests. Variation among mosquito genotypes in per cent response to honey, single human hosts, and single guinea pig hosts was assessed using one-way ANOVA followed by post hoc Tukey's HSD tests. Preference index variation in the DEET repellency assay and the nylon sleeve assay also were analysed in this way. Variation in preference in the live host assay, which included 14 different human subjects, was analysed using a two-way ANOVA that tested for the effects of both genotype and human subject followed by post hoc Tukey's HSD tests for pairwise differences among genotypes. Statistical significance in the human host proximity assay (Fig. 5b), biting assay (Fig. 5c), and landing (Fig. 5d) assays was determined using Bonferroni corrected *t*-tests testing for differences between solvent and DEET for each genotype. Violin plots in Figure 5b-d were generated in R (version 2.15.0) using the *wvioplot* package with the violins clipped to the extent of the data and an adjust parameter of 2. In Figure 5d, the violin for *orco*<sup>5/16</sup> with DEET is further clipped at the y-axis maximum of 2.0, excluding a single outlier data point of 3.76. Statistical significance of genotypic differences in mortality under fasting conditions was assessed using both a log rank test and a Gehan-Wilcoxon test. Both were significant ( $p < 0.004$ ,  $df=6$ ,  $\chi^2 = 19.3$  and  $19.5$  respectively). To determine which curves were significantly different, pairwise log rank tests were used. Significance was determined using Bonferroni corrected *p* values. All EC50 curve fits and statistical tests were performed with



Prism 5 software (GraphPad Software, La Jolla, CA, USA), JMP software version 8 (SAS Institute, Inc.) or R software version 2.15.0 (<http://www.r-project.org/>).

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