

## ARTICLES

# Odorant reception in the malaria mosquito *Anopheles gambiae*

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The mosquito *Anopheles gambiae* is the major vector of malaria in sub-Saharan Africa. It locates its human hosts primarily through olfaction, but little is known about the molecular basis of this process. Here we functionally characterize the *Anopheles gambiae* odorant receptor (AgOr) repertoire. We identify receptors that respond strongly to components of human odour and that may act in the process of human recognition. Some of these receptors are narrowly tuned, and some salient odorants elicit strong responses from only one or a few receptors, suggesting a central role for specific transmission channels in human host-seeking behaviour. This analysis of the *Anopheles gambiae* receptors permits a comparison with the corresponding *Drosophila melanogaster* odorant receptor repertoire. We find that odorants are differentially encoded by the two species in ways consistent with their ecological needs. Our analysis of the *Anopheles gambiae* repertoire identifies receptors that may be useful targets for controlling the transmission of malaria.

Mosquitoes transmit many diseases, including malaria, which afflicts hundreds of millions of people each year<sup>1</sup>. The malaria burden is heaviest in sub-Saharan Africa, where the *A. gambiae* mosquito is the major vector. *A. gambiae* relies heavily on olfactory cues to identify its human hosts<sup>2–4</sup>, but the molecular basis of host-seeking behaviour is unknown.

Insects detect odours by means of olfactory receptor neurons (ORNs). The odorant specificities of many ORNs are conferred by the expression of individual odorant receptor genes<sup>5</sup>. A family of 79 *Or* genes has been identified bioinformatically in *A. gambiae*<sup>6,7</sup>. Two of these receptors have been characterized functionally<sup>8</sup> using an *in vivo* heterologous expression system, the ‘empty neuron’ system<sup>9</sup>, which has also been used to decode the *D. melanogaster* odorant receptor repertoire<sup>10–12</sup>. These results invited a systematic, functional characterization of the AgOr repertoire and a comparison between the receptor repertoires of these two species, which exhibit different olfactory-driven behaviours. *D. melanogaster* consumes fruit and is considered a generalist. *A. gambiae* has evolved an anthropophilic host-seeking olfactory response that allows it to find human blood-meals<sup>4</sup>. Little is known about how the odorant receptor repertoires of these species have adapted to meet their distinct ecological requirements.

## Functional expression of the AgOr repertoire

To investigate the molecular basis of odour reception in *A. gambiae*, we amplified the coding regions of 72 AgOr genes from olfactory organ complementary DNA of adult mosquitoes. We then expressed each AgOr in the ‘empty neuron’, a mutant ORN in *D. melanogaster* that lacks its endogenous odorant receptor<sup>9</sup>. Fifty of the seventy-two cloned AgOr receptors were functional in the empty neuron, conferring a regular, characteristic, spontaneous firing rate and exhibiting excitatory and/or inhibitory responses to odorant stimuli (Fig. 1a). This success rate (69%) is comparable to that for *D. melanogaster* antennal *Or* genes (77%) expressed in the empty neuron<sup>11</sup>.

The empty neuron system was previously demonstrated to be a high-fidelity expression platform for the *D. melanogaster Or* (*DmOr*) genes<sup>11</sup>. Because *A. gambiae* and *D. melanogaster* are separated by 250 million years of evolution<sup>13</sup>, we wanted to determine whether the empty neuron is also a faithful expression system for AgOr genes. One

of the few AgOrs that has been unequivocally mapped to a specific ORN in the mosquito is AgOr8, which in its endogenous neuron responded to seven- and eight-carbon-chain compounds among a panel of tested odorants<sup>14</sup>. We expressed AgOr8 in the empty neuron and found that its response profile closely resembled that of the endogenous neuron (Fig. 1b). We also generated dose-response curves for two ligands of AgOr8, 1-octen-3-ol and 1-hepten-3-ol (Supplementary Fig. 1), and found that the differential sensitivity to these ligands observed in the endogenous neuron<sup>14</sup> was maintained in the empty neuron. These results validate the empty neuron as a reliable heterologous expression system for AgOrs.

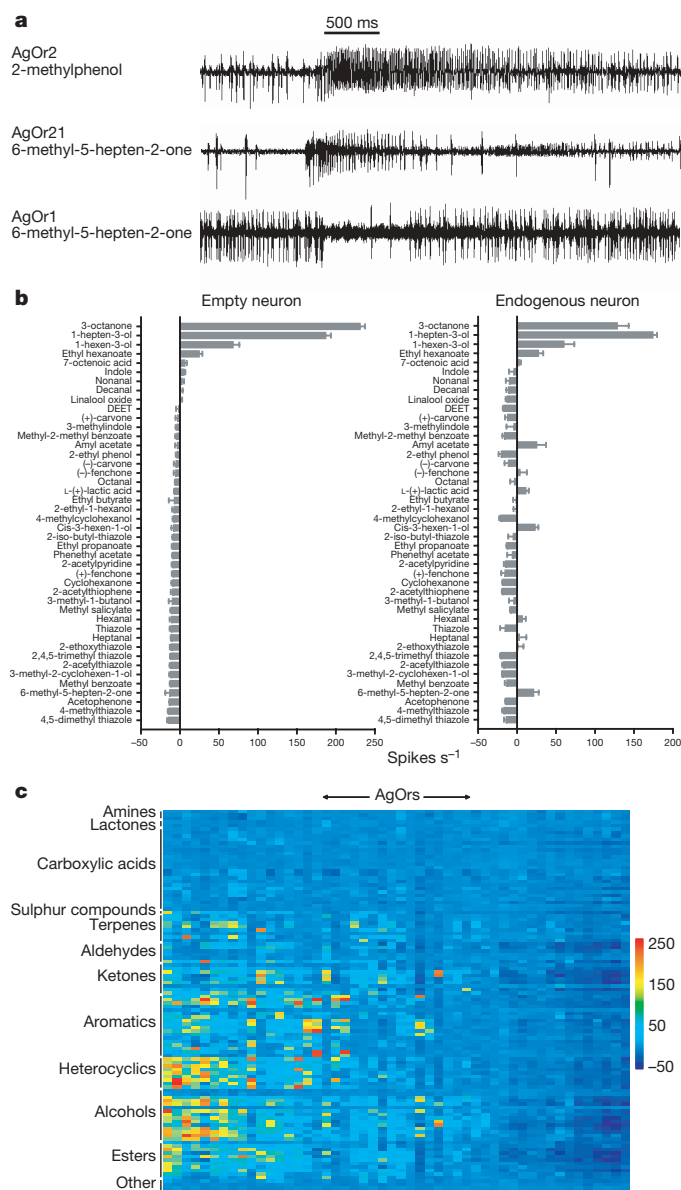
The 50 functional AgOrs were tested against a chemically diverse panel of 110 odorants, including components of human emanations and oviposition site volatiles (Supplementary Table 1). Of the 110 odorants, 53 were previously tested against the *D. melanogaster* antennal receptor repertoire in the empty neuron system<sup>12</sup>, permitting functional comparisons between the odorant receptor repertoires of the fruitfly and the mosquito.

We tested each of the functional AgOrs against the 110-odorant panel, generating a data set of 5,500 odorant–receptor combinations, with each combination tested  $n \geq 5$  times (Fig. 1c and Supplementary Table 2a–d). We found that individual receptors respond to subsets of odorants, and individual odorants activate subsets of receptors, consistent with a combinatorial model of odour coding<sup>12,15,16</sup>. Some receptors gave strong responses (defined as  $\geq 100$  spikes  $s^{-1}$ ) to many odorants, whereas other receptors are more selective (Supplementary Table 2a–d). These differences were visualized by generating a tuning curve for each receptor (Fig. 2). The breadths of the tuning curves were quantified according to their kurtosis value, a measure of the ‘peakedness’ of the distribution (Supplementary Table 3a, b). We found a continuum of AgOr tuning breadths ranging from broad to narrow, consistent with analysis of the *DmOr* repertoire<sup>12</sup>. We then considered the receptors at each extreme for insight into the molecular basis of odour recognition.

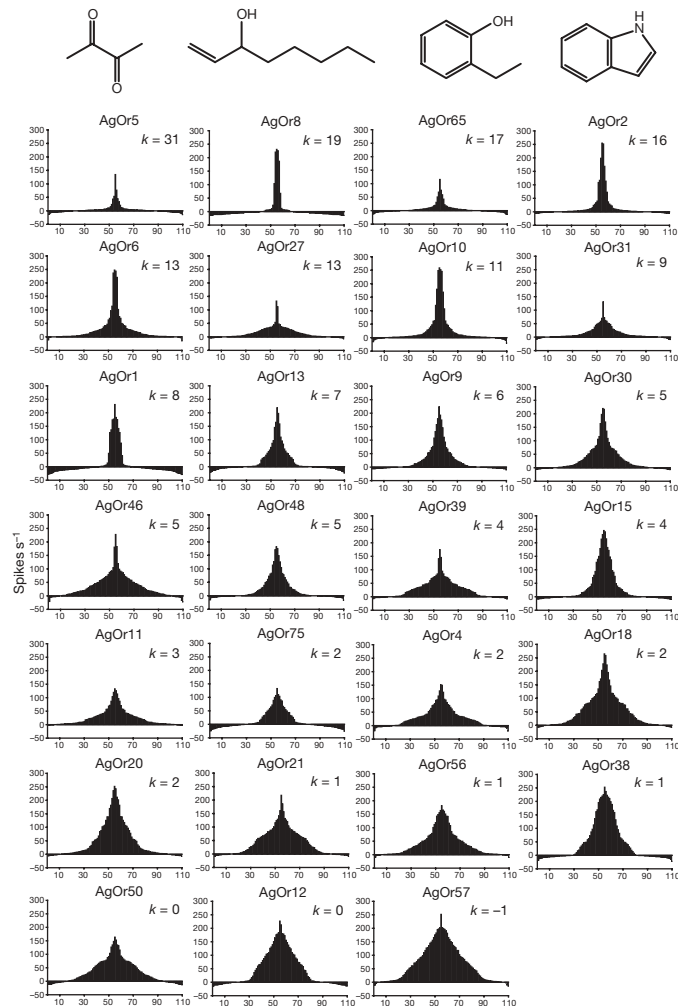
## Narrowly tuned receptors for salient odorants

Narrowly tuned receptors have been suggested to be specialist channels that carry information about odorants of high biological relevance<sup>17</sup>.

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**Figure 1 | Functional characterization of the AgOrs.** **a**, Extracellular recordings from the empty neuron expressing an AgOr. Top, excitatory response of AgOr2 to 2-methylphenol; middle, excitatory response of AgOr21 to 6-methyl-5-hepten-2-one; bottom, inhibitory response of AgOr1 to 6-methyl-5-hepten-2-one. Action potentials from both neurons housed in the sensillum can be observed and distinguished by amplitude; the larger-amplitude action potential, from the 'A' neuron, expresses the AgOr, whereas the smaller-amplitude action potential, from the 'B' neuron, expresses its endogenous *D. melanogaster* odorant receptor<sup>9</sup>. **b**, Left, odorant response profile of AgOr8 expressed in the empty neuron. Right, odorant response profile of the *A. gambiae* neuron that houses AgOr8 (adapted from ref. 14). All odorants were tested at a dilution of 10<sup>-2</sup>. The spontaneous firing rate and the responses to the diluent are subtracted from odorant responses in each panel. Of the 28 odorants that inhibited the spontaneous firing rate by 50% or more in the empty neuron<sup>45</sup>, 21 gave mean responses that were negative in the endogenous *A. gambiae* neuron. Error bars represent s.e.m. **c**, Heat map of responses of the 50 functional AgOrs to 110 odorants. Response intensity is colour-coded according to the continuous colour scale on the right, and represents the mean activity measured over a 0.5-s odorant-stimulation period. Receptors, odorants and numerical values are indicated in Supplementary Table 2.  $n = 5-6$ ; for odorants that elicit responses  $\geq 100$  spikes s<sup>-1</sup>,  $n = 6$ . Spontaneous activity and responses to diluent have been subtracted from response values. All odorants were tested at a 10<sup>-2</sup> dilution. Odorants containing both a phenol ring and an ester moiety are classified as aromatics; terpenes containing an ester moiety are classified as terpenes.



**Figure 2 | Tuning breadths of receptors.** Tuning curves for the AgOrs that respond strongly ( $\geq 100$  spikes s<sup>-1</sup>) to at least one odorant on the panel. The 110 odorants are arranged along the x-axis according to the strength of the response they elicit from each receptor. The odorants that elicit the strongest responses are placed near the centre of the distribution; those that elicit the weakest responses are placed near the edges. The order of odorants is therefore different for each receptor. The kurtosis value,  $k$ , a statistical measure of 'peakedness', is shown for each plot. Structures of odorants that elicit strong responses from the most narrowly tuned AgOrs (2,3-butanedione (AgOr5); 1-octen-3-ol (AgOr8); 2-ethylphenol (AgOr65); and indole (AgOr2)) are shown above the receptors they activate.

Consistent with this hypothesis, the most narrowly tuned AgOrs are robustly excited by odorants with high biological salience. Among the receptors that respond strongly to at least one odorant of the panel, the most narrowly tuned are AgOr2, AgOr8, AgOr5 and AgOr65. AgOr2 is narrowly tuned to a small set of aromatics including indole, which was found to constitute nearly 30% of the volatile headspace of human sweat<sup>18</sup>. AgOr8 responds strongly to 1-octen-3-ol, a human volatile that is a strong attractant for several species of mosquito<sup>4</sup>. AgOr5 is tuned to 2,3-butanedione, which is a metabolic byproduct of human skin microflora<sup>19</sup>. AgOr65 responds strongly to 2-ethylphenol, which is found in the urine of many animals<sup>20,21</sup>. We found no mosquito receptors narrowly tuned to esters or aldehydes—odorants that dominate the headspace of many fruits<sup>22,23</sup>. By contrast, among the most narrowly tuned receptors in the fruitfly, in most cases the strongest responses are to esters (DmOR85A, DmOR59B and DmOR67C) or to a terpene that contains an ester group (DmOR82A)<sup>12</sup> (Supplementary Table 3b).

Some of the narrowly tuned AgOrs, in addition to responding strongly to odorants with high biological salience, respond with high sensitivity to these odorants. AgOr8 and AgOr2 respond to concentrations of 1-octen-3-ol and indole that range over more than four

orders of magnitude and have response thresholds that lie between a  $10^{-7}$  and a  $10^{-6}$  dilution (Supplementary Fig. 2).

Broadly tuned receptors lie at the other end of the AgOr distribution. It is possible that these receptors act in signalling the presence of odorants but not in specifically identifying or discriminating among them. We found that in *A. gambiae*, all strong responses to esters and aldehydes are conferred by broadly tuned AgOrs (all have kurtosis values less than the mean; Supplementary Tables 2b and 3a).

### Salient odorants that activate specific receptors

'Odorant tuning curves', the reciprocal of receptor tuning curves, were also generated (Fig. 3, Supplementary Table 3c and Supplementary Fig. 3). For each odorant we plotted the responses of the 50 receptors along the *x*-axis, placing the strongest response at the centre. Interestingly, the five odorants with the most narrow response distributions are all highly relevant to mosquito ecology. 3-methylindole is an oviposition site volatile that induces egg-laying and ORN responses in *Culex* mosquitoes<sup>24,25</sup>. Indole is another oviposition site volatile<sup>24,26,27</sup>, in addition to being a major component of human emanations that is found in both sweat<sup>18</sup> and human breath<sup>28</sup>. Geranyl acetate and citronellal are emitted from plants that are repellent to *A. gambiae*<sup>29,30</sup>. Dimethylsulphide is emitted in human breath<sup>31</sup> and is attractive to the *Aedes aegypti* mosquito<sup>32</sup>.

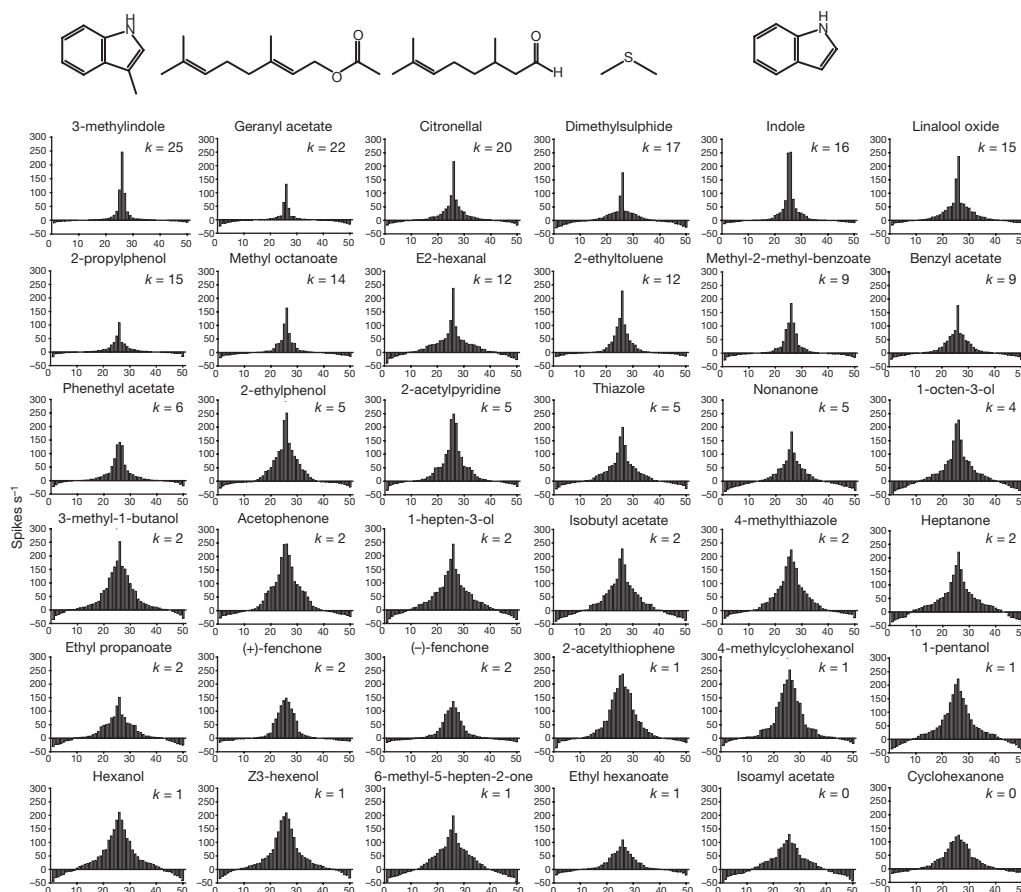
*D. melanogaster* odorant tuning curves were constructed from earlier data<sup>12</sup> to identify odorants that similarly strongly activate a small number of receptors (Supplementary Table 3d). In contrast to the *A. gambiae* tuning curves, many of the odorants with high kurtosis

values are esters, the dominant chemical class in fruit emanations<sup>23</sup>. Together, the mosquito and fruitfly odorant tuning curves support a model in which odorants of particular biological relevance are coded by a small number of channels.

Taken together, these results indicate that odorant and receptor tuning analyses provide complementary approaches for identifying receptors and odorants that are important for innate insect behavioural responses. We note with special interest that in *A. gambiae*, one of the 'narrowly tuned' odorants activates one of the narrowly tuned receptors. AgOr2 is strongly activated by indole, the odorant that constitutes almost 30% of the volatile headspace of human sweat<sup>18</sup>.

### Diverse temporal dynamics of receptor responses

DmOrs were previously shown to yield responses that vary widely in their temporal characteristics, and the temporal dynamics were specific to the odorant–receptor combination<sup>11,12</sup>. To investigate the temporal dynamics of responses from AgOrs, we generated peristimulus time histograms for several odorant–receptor combinations (Supplementary Fig. 4). As was observed with DmOrs, we find a diversity of temporal dynamics. Some odorants, including the human volatiles 1-octen-3-ol and indole, were capable of generating tonic responses that persisted throughout the 3-s analysis period. Linalool oxide also generated a prolonged response. These odorants generated phasic responses from other receptors. The diversity of these responses suggests that temporal features may be a rich source of information about odorant identity.

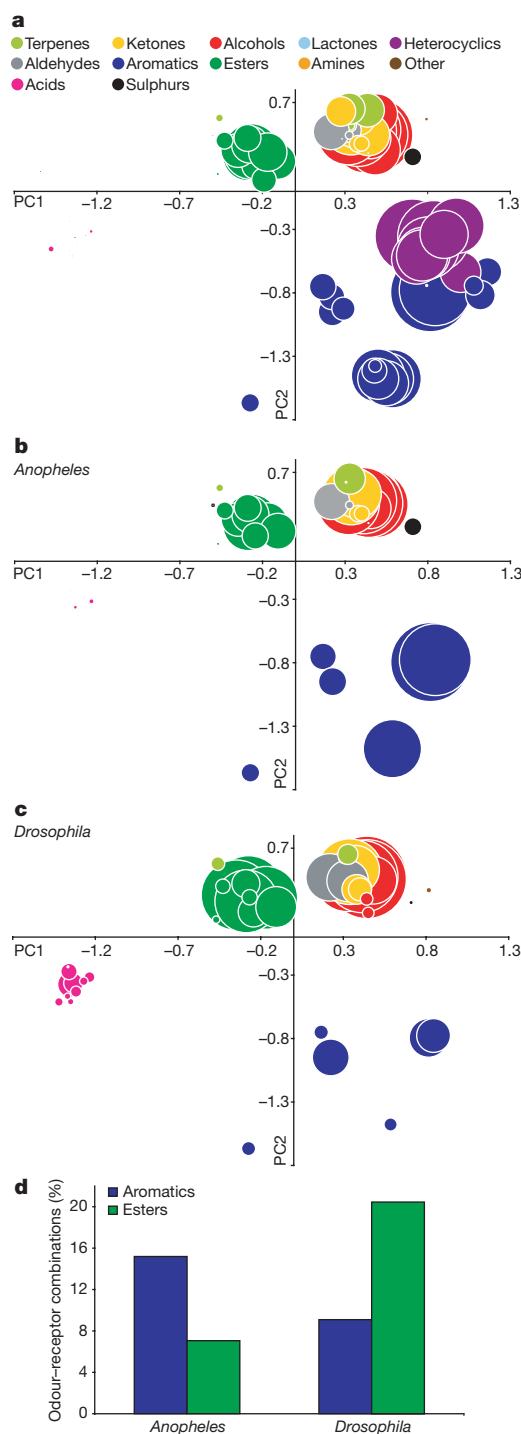


**Figure 3 | Odorant tuning curves.** The responses of the 50 AgOrs are ordered along the *x*-axis according to the magnitude of the response they generate for each odorant. The receptor with the strongest response is placed at the centre of the distribution; those that have the weakest responses are at the edges. The order of receptors is therefore different for each odorant. The kurtosis value is indicated in each graph. Only odorants that generate a strong response ( $\geq 100$  spikes  $s^{-1}$ ) from at least one receptor are shown. At

the top of the panel are the structures of several odorants that generate strong responses from just one or two receptors, shown above the corresponding graph. Shown here are tuning curves for the 12 most narrowly tuned odorants, the 12 most broadly tuned odorants, and 12 representative odorants of intermediate tuning breadth. Tuning curves for 25 other odorants are shown in Supplementary Fig. 3.

## How the mosquito covers odour space

An intriguing question is how the chemical world is represented by the AgOrs. Excitatory activity is not distributed evenly across the odorant panel (Fig. 1c and Supplementary Table 2a). For example, 28% of the strong responses were generated by heterocyclics, which constitute only 8% of the odorants in the panel. However, chemical class is only one descriptor of molecular identity. To represent the structural diversity among odorants more fully we adapted a recently developed odorant metric<sup>33</sup>. This metric is derived from an optimized set of 32 molecular descriptors, including functional group, carbon chain length, and other physicochemical properties, which provide the basis of a 32-dimensional coordinate system. Each odorant can be mapped to a unique location in this multidimensional space. Odorants that are structurally similar lie close together in the space, whereas odorants



that are structurally dissimilar lie far apart. To visualize this space we applied principal components analysis (PCA) to project it into two dimensions (Supplementary Fig. 5a). As shown by the aromatics, odorants can be of the same chemical class yet map far apart.

Having mapped the odorants of the panel into this chemically defined odour space, we then asked how the AgOr repertoire covers the space. To illustrate the responses elicited by each odorant we generated a bubble plot, in which the location of each bubble indicates odorant identity, and the size of each bubble represents the magnitude of the response to that odorant, summed across all receptors and measured in total spikes  $s^{-1}$  (Fig. 4a).

The AgOr repertoire is sensitive to a broad region of the odour space. However, the responses are not of uniform magnitude across the space. The odorants in the region of the space that is occupied by heterocyclics (purple), for example, elicit greater responses than the odorants in the region that is home to esters (dark green), and much greater responses than those in the region inhabited by carboxylic acids (pink). The AgOr repertoire may have evolved particular sensitivity to certain regions of odour space, such as the region containing aromatics, some of which are major components in human emanations<sup>18,34–40</sup>, and heterocyclics, a chemical class that includes volatiles proposed to promote oviposition behaviour<sup>27</sup>. Such enhanced sensitivity could reflect the insect's investment in detecting and discriminating among chemicals of these classes.

We also investigated the distribution of inhibitory responses across the *A. gambiae* receptor repertoire, which are not visualized in the odour space described earlier. As was observed across the *D. melanogaster* receptor repertoire<sup>12</sup>, most odorants elicit at least one inhibitory response, and most receptors are inhibited by at least one odorant (Supplementary Table 2a).

## *A. gambiae* and *D. melanogaster* odour spaces

We next asked whether the *A. gambiae* and *D. melanogaster* Or repertoires differ in their coverage of odour space. As an initial means of addressing this issue, we considered the 53 odorants that were tested against both the AgOr and DmOr repertoires<sup>12</sup> and constructed odour spaces of the type described earlier for each receptor repertoire.

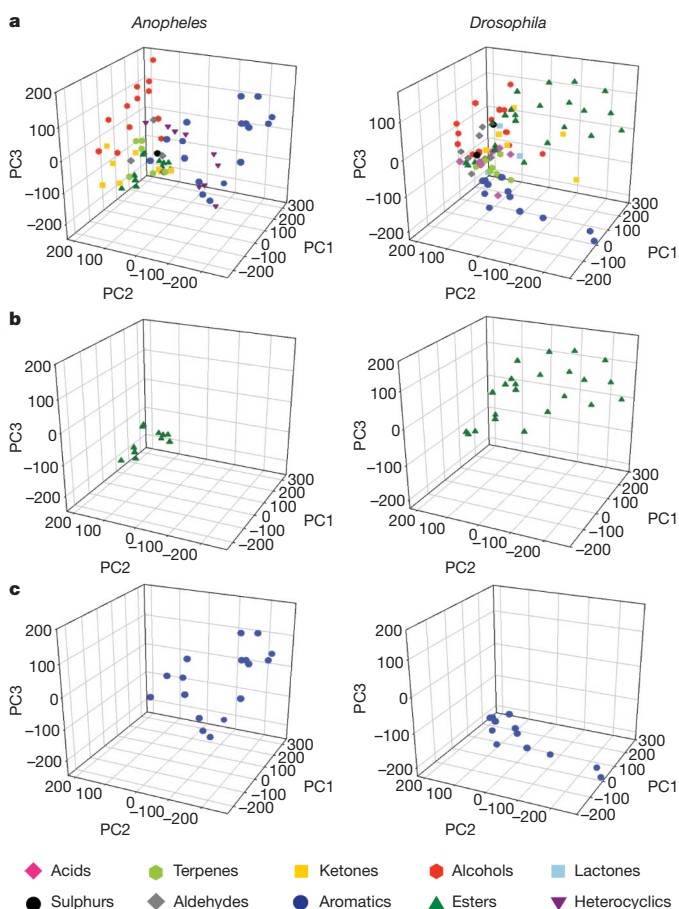
The two species differed in their relative coverage of odour space. The mosquito allocated greater relative coverage to the aromatics (Fig. 4b; dark blue in bottom-right quadrant). By contrast, the fly devoted greater relative activity to some of the esters (dark green) and one of the two aldehydes that were compared (grey) (Fig. 4c and Supplementary Fig. 5b). We then compared the two species with respect to the strong responses ( $\geq 100$  spikes  $s^{-1}$ ). In the mosquito, 15% of the receptor–aromatic combinations yielded strong responses, compared to 7% of the receptor–ester combinations. By contrast, in the fly, 9% of the receptor–aromatic combinations

**Figure 4 | Distribution of responses across a physicochemical odour space.** **a**, Bubble plot of the responses generated by the AgOr repertoire to each of the 110 odorants. The size of the bubble scales with the sum of spikes across all receptors that exhibit at least one response  $\geq 50$  spikes  $s^{-1}$  from at least one odorant on the panel. Each odorant is plotted and colour-coded according to functional group, except for 7-octenoic acid and 2-oxohexenoic acid. Shown are the first two principal components (PC) of the 32-dimensional physicochemical space (adapted from ref. 33). Descriptors were normalized. **b**, **c**, Comparison of responses generated by the AgOrs (**b**) and DmOrs (**c**) to the set of 53 odorants that were tested against both receptor repertoires. Size of the bubble corresponds to the sum of spikes across all receptors that exhibit at least one response  $\geq 50$  spikes  $s^{-1}$  from at least one odorant on the panel. Responses were normalized to the sum of spikes elicited by all 53 odorants across all AgOrs or all DmOrs that exhibit at least one response  $\geq 50$  spikes  $s^{-1}$  from at least one odorant on the panel. **d**, The percentage of odorant–receptor combinations that generate strong responses ( $\geq 100$  spikes  $s^{-1}$ ) within the ester and aromatic classes for *A. gambiae* and *D. melanogaster*. Only responsive receptors (those yielding a response  $\geq 50$  spikes  $s^{-1}$  to at least one odorant) were considered.

yielded strong responses, compared to 20% of the receptor–ester combinations (Fig. 4d).

We next considered whether the species differed with respect to another kind of odour space—a biological odour space that relates odorants on the basis of the primary sensory signals they generate. We created a space in which each axis represents the response magnitude (in spikes  $s^{-1}$ ) for one odorant receptor, as described previously<sup>12</sup>. Odorants that elicit similar patterns of activity across the receptor repertoire map close together. Odorants that are close may be similar in their perceptual qualities, and may be more difficult for the animal to discriminate because they generate similar patterns of ORN activity. Experiments conducted with *Drosophila* larvae have provided evidence to support a relationship between such odour space distances and perception<sup>10</sup>.

We constructed such spaces for the mosquito and the fly and depicted them in three dimensions by applying PCA (Fig. 5a). Odorants of the same chemical class tend to cluster together (Fig. 5a), as previously observed in the fly<sup>12</sup>. However, some chemical classes were differentially distributed in the odour spaces of the two insects. Esters were more widely distributed in *D. melanogaster* space than in *A. gambiae* space, whereas aromatics were more widely distributed in *A. gambiae* space (Fig. 5b, c). To quantify these differences,



**Figure 5 | Distribution of odorants in a receptor activity-based odour space.** First three principal components of a receptor activity-based odour space. **a**, Left, *A. gambiae* odour space. Right, *D. melanogaster* odour space (adapted from ref. 12). All odorants tested against a receptor set were considered. Odorants are colour-coded according to functional group. **b**, Only esters are shown. Left, *A. gambiae* odour space; right *D. melanogaster* odour space. **c**, Only aromatics shown. Left, *A. gambiae* odour space; right *D. melanogaster* odour space. Mean inter-odorant distance for the set of esters tested against both receptor sets is  $254 \pm 12$  spikes  $s^{-1}$  for the AgOrs; and  $353 \pm 13$  spikes  $s^{-1}$  for the DmOrs ( $P < 0.05$ ;  $t$ -test). Mean inter-odorant distance for the set of aromatics tested against both receptor sets is  $406 \pm 29$  for the AgOrs; and  $321 \pm 17$  for the DmOrs ( $P < 0.05$ ;  $t$ -test).

we calculated the Euclidean distance for every pair of odorants with the same functional group, within the odour space of each species. We found that the mean inter-odorant distance for esters is significantly higher for *D. melanogaster* than *A. gambiae*, whereas the mean inter-odorant distance for aromatics is higher for *A. gambiae* ( $P < 0.001$  for esters,  $P = 0.01$  for aromatics, Mann–Whitney); there were no differences in distances among alcohols or ketones, the other groups that could be compared. These results indicate that mosquitoes may be better able than fruitflies to discriminate among aromatics, whereas fruitflies may be better able to discriminate among esters, perhaps reflecting the biological relevance of these classes of compounds to the animals.

We then asked whether the functional differences between the mosquito and fruitfly Or repertoires are due to a particular clade of mosquito or fly receptor. When the AgOr family was first identified, phylogenetic analysis revealed a clade of *A. gambiae* odorant receptors with no close *D. melanogaster* relatives, and a clade of *Drosophila* odorant receptors with no close *A. gambiae* relatives<sup>6,7</sup> (see also Supplementary Fig. 6). From these observations, the question arises as to whether these species-specific clades of receptors respond to odorants of a particular kind. We used matrix analysis to generate a dendrogram of receptors (Supplementary Fig. 7), and PCA to create a ‘receptor space’, based on the responses of the receptors to odorants (Supplementary Fig. 8). In neither case did we observe clustering of receptors of the species-specific clades. Thus, the observed functional differences between the *A. gambiae* and *D. melanogaster* odorant receptor repertoires may reflect evolutionary changes distributed across the receptor repertoires rather than concentrated in a specific branch of the phylogenetic tree.

We note finally that no AgOr showed even a modest response of 50 spikes  $s^{-1}$  to any carboxylic acid or any amine in our system. By contrast, some DmOrs respond strongly to certain carboxylic acids<sup>12,41</sup>. Recently, a set of variant-ionotropic glutamate receptors that respond to amines and carboxylic acids have been identified in *D. melanogaster*<sup>42</sup>. Receptors of this class may detect these compounds in *A. gambiae*.

## Discussion

Here we have functionally characterized the AgOr repertoire of odorant receptors from the mosquito *A. gambiae*. The olfactory system of *A. gambiae* allows the insect to locate human blood-meal hosts, thereby facilitating the transmission of malaria. We have identified individual receptors that respond robustly to human volatiles and may be central to the process by which the mosquito identifies its human hosts.

Notably, some receptors that respond to human odorants are narrowly tuned and highly sensitive. Reciprocally, some notable odorants elicit strong responses from one or a few receptors. These highly focused relationships between certain odorants and receptors suggest a role for specific transmission channels in guiding the animal’s behaviour. A recent study of the *D. melanogaster* antennal lobe documented lateral inhibitory interactions that increased with greater total ORN input<sup>43</sup>. Odorants that excite few receptors in the mosquito may produce signals that suffer less inhibition and enjoy more saliency. Another study found that ablation of either of two narrowly tuned ORN classes impaired behavioural attraction to their cognate odorants<sup>44</sup>.

Because our analysis was conducted using the same expression system as our previous study of *D. melanogaster* odorant receptors, it provided a unique opportunity to compare the Or repertoires of two species that belong to the same order but that exhibit different olfactory-guided behaviours. An outstanding question in the field of olfaction is how an organism’s ecology shapes the function of its odorant receptor repertoire. A full answer to this question requires the functional characterization of entire receptor repertoires. We found that the two species show different coverage of a chemically defined odour space. Certain classes of odorants are differentially

distributed in a biologically defined odour space of each species. These differences suggest the evolution of olfactory acuity and discriminatory power consistent with the ecological needs of each species. These evolutionary changes seem to have occurred over the odorant receptor repertoire as a whole, as opposed to having been effected by the emergence of a species-specific receptor clade.

The results may have implications for the control of malaria, one of the world's most devastating diseases. Screens for activators and inhibitors of selected receptors may identify compounds that attract mosquitoes into traps, interfere with their navigation, or repel them.

## METHODS SUMMARY

AgOr cDNAs were cloned by standard procedures and expressed in the empty neuron as described previously<sup>8,14</sup>. Extracellular single-unit physiology was performed as described previously<sup>9,11</sup>. Physicochemical odour space was constructed using the set of 32 optimized DRAGON descriptors<sup>33</sup>, which were normalized.

**Full Methods** and any associated references are available in the online version of the paper at [www.nature.com/nature](http://www.nature.com/nature).

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**Supplementary Information** is linked to the online version of the paper at [www.nature.com/nature](http://www.nature.com/nature).

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## METHODS

**Cloning of the AgOrs.** Five-day-old laboratory-maintained *A. gambiae* mosquitoes of the Suakoko strain were cold anaesthetized and the antennae, maxillary palpa, and proboscises were dissected by hand on a chill table. RNA was prepared with RNeasy (QIAGEN) according to the manufacturer's instructions. The RNA preparation was used for oligo dT-primed cDNA synthesis with Superscript II Reverse Transcriptase (Invitrogen) for the generation of templates for subsequent PCR reactions. Negative control samples with no reverse transcriptase were included in each cDNA synthesis and subsequent PCR analysis. PCR was performed with a Mastercycler Gradient (Eppendorf) under the following conditions: 94 °C for 5 min; 32–40 cycles of 94 °C for 30 s, 55–60 °C for 30 s (annealing temperature varied depending on primer pair), 72 °C for 60–90 s; and 72 °C for 7 min. PCR amplification products were separated on a 1.0% agarose gel and were cloned into the Gateway pENTR entry vector (Invitrogen) or pGEM-TEasy (Promega) and verified by sequencing. Sequences of at least two independent clones were obtained for each *Or* and compared to verify polymorphisms as such rather than PCR errors. Sequence discrepancies were resolved by a second PCR reaction. We amplified 72 AgOrs, in addition to AgOr7, the *A. gambiae* orthologue of the atypical *D. melanogaster* odorant receptor gene *Or83b*<sup>46</sup>. Four (AgOr37, AgOr40, AgOr52 and AgOr58) of the remaining six AgOr genes that were not amplified from adult tissue are expressed in the larval stage<sup>47</sup>, and the other two may be artefacts of gene annotation.

**Drosophila stocks and transgenes.** The ab3A mutant flies and *Or22a-GAL4* constructs were described previously<sup>9,11</sup>. To generate *UAS-Or* constructs, the pUAST vector (C. G. Warr) was adapted to generate a Gateway-compatible destination vector (Invitrogen). Recombination reactions using LR Clonase (Invitrogen) were performed with the Gateway pENTR entry clones (Invitrogen) and the modified pUAST vector. For those amplification products cloned into pGEM-TEasy, subcloning into unadapted pUAST was performed using the restriction enzymes BglII, KpnI and NotI (New England Biolabs). An exception was AgOr53, which was cloned into the pNmyc-UAST vector (C. G. Warr) in frame with the start codon and three copies of the Myc-tag coding sequence. The resulting protein had an amino-terminal Myc tag. No other receptors had epitope tags.

**Electrophysiology.** Extracellular single-unit recordings were performed essentially as described previously<sup>11</sup>. Odorant stimuli were prepared in Pasteur pipettes as described previously<sup>11</sup>. Chemicals were of the highest purity available (Sigma-Aldrich). Seven-octenoic acid and *cis/trans*-3-methyl-2-hexenoic acid were synthesized by Richman Chemical. All chiral chemicals were racemic mixtures with the exception of (+)-carvone, (–)-carvone, (+)-fenchone, (–)-fenchone and L(+)-lactic acid. Ammonia, cadaverine, putrescine, acetic acid, propanoic acid, butanoic

acid, isobutyric acid, isovaleric acid, L(+)-lactic acid and 2-oxohexenoic acid were diluted in H<sub>2</sub>O. Hexadecanoic acid, octadecanoic acid and 5 $\alpha$ -androst-3 $\alpha$ -ol, were diluted in ethanol. All other odorants were diluted in paraffin oil (Fluka). Liquid odorants were diluted in solvent to a concentration of 10<sup>–2</sup> (v/v), and solid odorants were dissolved, 50 mg in 5 ml solvent.

Stimuli were presented by placing the tip of the pipette through a hole in a tube carrying a purified air stream (32 ml s<sup>–1</sup>) directed at the fly and administering a pulse of charcoal-filtered air (3.2 ml s<sup>–1</sup>) through the pipette containing the odorant. Pulse duration was 500 ms. Stimuli were used for a maximum of four presentations. Responses were quantified by subtracting the number of impulses in 500 ms of unstimulated activity from the number of impulses in the 500 ms after odorant stimulation, after a 150 ms delay to allow the odorant to travel down the airstream. Responses to diluents were also subtracted. For each odorant, each recording was from a separate sensillum, with no more than three sensilla analysed per fly. Recordings were obtained from flies between 4 and 14 days old.

A panel of six odorants previously tested against the ab3B neuron<sup>11</sup> were re-tested in this study to control for possible differences between electrophysiology rigs; none of the responses were significantly different ( $P \geq 0.12$  in all cases, *t*-test).

**Data analysis.** PCA and hierarchical cluster analysis were performed using PAST, a statistics program (<http://folk.uio.no/ohammer/past/>) as described previously<sup>12</sup>. Physicochemical odour space was constructed using the set of 32 optimized DRAGON descriptors<sup>33</sup> (Talete, srl, DRAGON for Windows, version 5.5, 2007, <http://www.talete.mi.it/>). Descriptors were normalized. The 12 odorants that generate net negative (inhibitory) responses (1-chlorododecane, 3-methyl-2-hexenoic acid, cadaverine, *cis*-9-octadecanoic acid, delta-decalactone, hexadecanoic acid, nonanoic acid, octadecanoic acid, octanal, octanoic acid, putrescine and tridecanoic acid) are not shown in the bubble plots of Fig. 4. To generate odour spaces and for cluster analyses, we removed from analysis receptors that did not respond to any odorant on the panel with a response  $\geq 50$  spikes s<sup>–1</sup> and odorants that did not elicit any responses  $\geq 50$  spikes s<sup>–1</sup> (before solvent responses were subtracted) unless otherwise noted. Error bars represent s.e.m. unless otherwise noted. Phylogenetic analysis was performed with MEGA 4.0.2, using a Neighbour-joining algorithm and 500 replications. Peristimulus time histograms were generated using IGOR Pro 6.0 (Wavemetrics).

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