# The molecular and cellular basis of olfactory-driven behavior in *Anopheles gambiae* larvae

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**The mosquito *Anopheles gambiae* is the principal Afrotropical vector for human malaria. A central component of its vectorial capacity is the ability to maintain sufficient populations of adults. During both adult and preadult (larval) stages, the mosquitoes depend on the ability to recognize and respond to chemical cues that mediate feeding and survival. In this study, we used a behavioral assay to identify a range of odorant-specific responses of *An. gambiae* larvae that are dependent on the integrity of the larval antennae. Parallel molecular analyses have identified a subset of the *An. gambiae* odorant receptors (*AgOrs*) that are localized to discrete neurons within the larval antennae and facilitate odor-evoked responses in *Xenopus* oocytes that are consistent with the larval behavioral spectrum. These studies shed light on chemosensory-driven behaviors and represent molecular and cellular characterization of olfactory processes in mosquito larvae. These advances may ultimately enhance the development of vector control strategies, targeting olfactory pathways in larval-stage mosquitoes to reduce the catastrophic effects of malaria and other diseases.**

malaria | olfaction | signal transduction | odorant receptors

Sensitivity and the ability to respond to a wide range of olfactory cues are essential for many behavioral processes that mediate the vectorial capacity of *Anopheles gambiae* and other disease-carrying mosquitoes (1). Although there is a growing body of knowledge of the adult *An. gambiae* olfactory system, there is a paucity of information as to the molecular and cellular basis of olfaction in larval stages, in which it may be of potential importance in disease control. Paradoxically, despite being one of the historically most successful strategies for mosquito control (2) and prevention of human malaria, the targeting of mosquito larvae or larval habitats around human dwellings is sparsely implemented in Africa and other malaria-endemic regions (3). Furthermore, the simplicity of insect larval olfactory systems makes them excellent models to study olfactory signal transduction and coding. Indeed, the arbovirus vector mosquito *Aedes aegypti* expresses 24 odorant receptor (*OR*) genes in the larval antenna, 15 of which are larval specific (4). Elegant work in the *Drosophila melanogaster* model has detailed larval behavioral responses and characterized 25 ORs that are expressed in 21 olfactory receptor neurons (ORNs) in each of the two dorsal organs, which constitute the olfactory apparatus of the fly larva (5–7).

In this study, we designed and used a simple olfactory-based assay to carry out an initial characterization of *An. gambiae* larval behavioral responses to a range of natural and synthetic chemical stimuli. Consistent with olfactory function, ablation of the larval antennae specifically eliminated these behavioral responses, and molecular approaches identified a set of larval *AgOr*s, which were in some cases larval specific and the transcripts of which were mapped to a distinctive population of ORNs within the larval antennae. Functional analyses of larval AgORs were then carried out in *Xenopus* oocytes, validating their roles as bona fide OR proteins and demonstrating that larval AgORs are composed of both broadly and narrowly tuned receptors. These studies begin to define the odor space as well as the mechanistic basis of olfaction and olfactory-driven behavior in pre-adult stage *An. gambiae*.

## Results

**Ultrastructure of the *An. gambiae* Larval Antennae.** The antennae of *An. gambiae* larvae are bilaterally symmetrical and project as a single extension anteromedially from the lateral surface of the head [supporting information (SI) Fig. S1*A*]. Dipteran larvae share homologous chemosensory structures on their antennae, including a sensory cone and a peg organ, which are considered to be olfactory and gustatory, respectively (8). We used SEM to identify organs that resemble both the sensory cone and the peg organ, in addition to other setae residing on the distal tip of the antenna (Fig. S1 *B* and *C*). Not surprisingly, the number and morphology of larval antennal terminal structures were consistent among individuals and throughout larval instars, as well as similar to those described for *Ae. aegypti* (9).

Externally, the sensory cone has a finely ridged surface and is apparently aporous (Fig. S1*D*). Vacuoles were observed near the base of the cone, similar to those found in *Ae. aegypti*, that may function in lipid-soluble odor detection (9). The *An. gambiae* sensory cone was observed to be innervated by the dendrites of ≈12 bipolar neurons (Fig. S1 and Fig. 1*A*). All of these neurons have been shown to express AgOR7 (10), the functional ortholog of DmOr83b (11), which is required in the dorsal organ of *D. melanogaster* for larval olfaction (5). Given its structural similarities with other larval olfactory organs, we propose that the sensory cone is the principle olfactory organ in *An. gambiae* larvae.

***An. gambiae* Larvae Respond to Natural and Synthetic Odorants.** The logical output of the larval olfactory system is to generate distinct behavioral responses. To begin to understand the relationship between larval olfactory inputs and behavioral outputs in *An. gambiae*, we used a simple olfaction-based assay that tests the ability of *An. gambiae* second and third instar larvae to respond to source dilutions of a panel of 33 natural and synthetic odorant stimuli (Fig. 2*A*, *Methods*, and Table S1). Of the 33 odorants tested, *An. gambiae* larvae displayed significant responses to 11 (Fig. 2). Although the limits of our current analyses preclude a detailed characterization of the larval behavioral patterns, it is noteworthy that most of the odorants that elicited behavioral responses were aromatics, and that all of the cresols tested—2-methylphenol (*o*-cresol), 3-methylphenol (*m*-cresol) and 4-methylphenol (*p*-cresol)—manifested positive performance indices (PIs) at source dilutions as low as $10^{-5}$ (Fig. 2 *B–D*). At the other end of the behavioral spectrum, acetophenone evoked significantly negative PIs from *An. gambiae* larvae at similarly high source dilutions (Fig. 2*E*). Indole, another aromatic compound, induced variable responses in a concentration-dependent manner, in that *An. gambiae* larvae responded strongly

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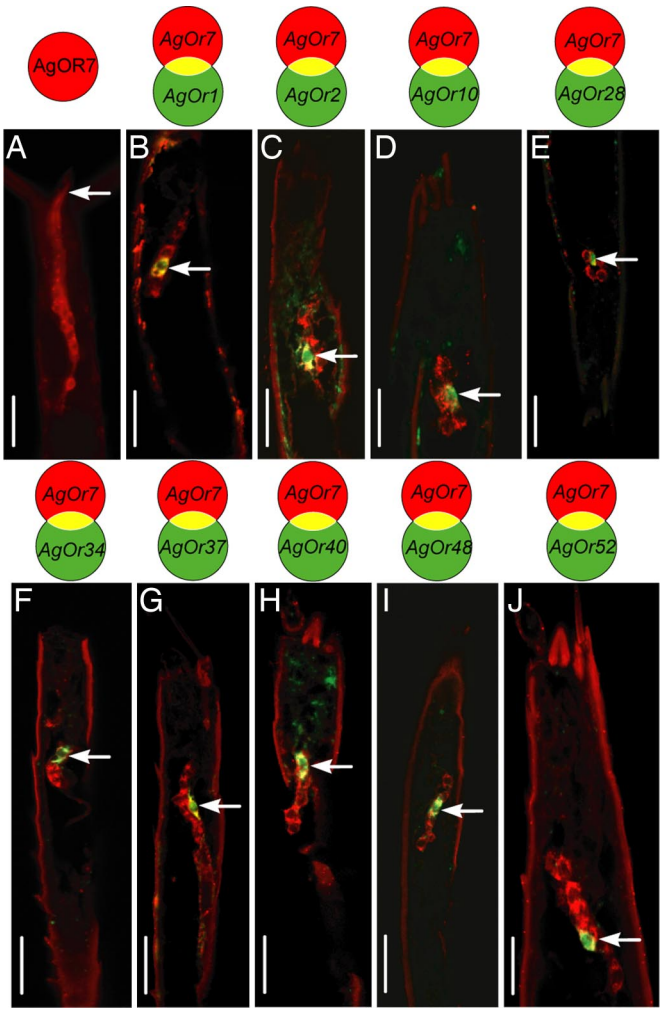


**Fig. 1.** Expression of *AgOr* genes in the larval antenna. (*A*) Whole-mount staining of *An. gambiae* larval antennae with AgOR7 antibody. The arrow indicates the dendrites projecting into the sensory cone. (*B–J*) *AgOr* FISH on 8-$\mu$m section results revealed that each individual conventional *AgOr* is solely coexpressed with *AgOr7* in a single larva OSN. Arrows indicate the individual neuron (yellow) with *AgOr7* (red) and one conventional *AgOr* (green) coexpressed. (Scale bar, 25 $\mu$m.)

to a $10^{-4}$ M source solution, although largely avoided higher ($10^{-2}$ M) concentrations (Fig. 2*F*). 1-Octen-3-ol, a well established odorant cue for adult mosquitoes that has been isolated from human and cattle odors (12), was responsive only at a single ($10^{-4}$) source dilution, with an average PI value of +0.34 (Fig. 2*G*). Several acids, including isovaleric acid, which has been shown to act as a strong attractant for adult *An. gambiae* (13), failed to evoke any statistically significant behavioral effects in larvae at the four different dilutions tested (Fig. 2*K*).

To better assess responses to potential larval food sources, yeast and two amino acids—methionine and phenylalanine, which have been shown to attract *Culex quinquefasciatus* larvae (14)—were used in our behavioral paradigm. *An. gambiae* larvae responded with significantly positive PIs to yeast across a range of source concentrations (Fig. 2*I*); however, apart from a potential avoidance response to 1.56 mg/ml phenylalanine, *An. gambiae* showed no preference to either of the amino acids (data not shown). In addition, the larvae were observed to manifest modest positive PIs in response to the sources of heterocyclic aromatic compounds carvone and thiazole at $10^{-5}$ dilutions but were indifferent when tested against lower dilutions (data not shown). Interestingly, the widely used insect repellent *N*,*N*-diethyl-m-toluamide (DEET), which has recently been shown to target olfactory pathways (15), consistently evoked dose-dependent and highly significant negative PIs at dilutions of $10^{-4}$ or less (Fig. 2*J*).

To provide additional evidence that the behavioral responses we observed are indeed mediated by the larval olfactory system, an antennal ablation study was carried out. Here, both antennae were carefully removed, and the larvae were allowed to recover under normal conditions for 1 day before behavioral analyses. Moreover, to control for potential artifactual effects of surgical injury, sham ablations of the larval maxilla were also undertaken. Importantly, with regard to overall mobility (distance traveled in 30 min), ablated larvae were indistinguishable from un-ablated controls (data not shown), indicating an absence of any general locomotor defect. Behavioral responses to two compounds that normally manifest strong but opposite reactions (2-methylphenol and DEET) were then examined. In each instance, ablation of the larval antennae resulted in a dramatic loss of odorant-driven behavioral responses (Fig. 2*L*). Of note, the PIs of larvae that had undergone maxilla ablations were statistically indistinguishable from those of unablated animals, providing strong correlative data linking olfactory input via the larval antenna to odor-driven behavioral output.

**Odorant Receptor Expression in Larval Olfactory Sensory Neurons.** At a molecular level, a set of putative ORNs have previously been identified on larval antenna on the basis of the expression of the nonconventional AgOR7 coreceptor (10). To determine the precise number of AgOR7$^+$ ORNs, whole-mount labeling of the larval antenna with the same antibody was carried out. A detailed examination of multiple ($n > 10$) preparations revealed that 12 ORNs were labeled with the AgOR7 antibody, and the dendrites of these neurons were observed to project into the sensory cone (Fig. 1*A*). Two different approaches were then taken to characterize any conventional *AgOr*s that were presumed to be coexpressed along with *AgOr7* in the larval ORNs. RT-PCR-based screens identified 12 conventional *AgOrs* that were consistently amplified from larval cDNA preparations. Of these, *AgOr*1, *AgOr*2, *AgOr*6, *AgOr*10, *AgOr*28, *AgOr*34, *AgOr*48, and *AgOr*49 have also been detected in adult olfactory appendages (16). Four larval *AgOrs*—*AgOr*37, *AgOr*40, *AgOr*52 and *AgOr*58—are likely to be larval-specific, as evidenced by no amplification being observed in similar experiments carried out using adult olfactory appendages (M. Rutler, G.W., H.W., and L.J.Z., unpublished observation). Based on the concordant number of larval *AgOrs* and ORNs, we used double *in situ* hybridization to examine whether each conventional larval *AgOr* is expressed together with *AgOr*7 in a single ORN. Of the 12 conventional *AgOrs*, nine (*AgOr1*, *AgOr2*, *AgOr10*, *AgOr28*, *AgOr34*, *AgOr37*, *AgOr40*, *AgOr48* and *AgOr52*) were coexpressed with *AgOr*7 (Fig. 1 *B–J*), whereas *AgOr6*, *AgOr49*, and *AgOr58* failed to generate consistent *in situ* signals. This is consistent with the relatively weak amplification of these *AgOr*s in semiquantitative RT-PCR studies (data not shown), suggesting they may be expressed at very low levels in the corresponding ORNs. Furthermore, an exhaustive examination of multiple antennal sections using probes for individual and pairs of larval *AgOrs* ($n > 5$) indicated that each conventional *AgOr* is expressed together with *AgOr7* in distinct and stereotypic larval ORNs (data not shown). For example, mixed probes of *AgOr34* and *AgOr37* always label distinct individual neurons, consistent with the lack of coexpression of these two *AgOr*s in the same neuron ($n = 4$, data not shown). Interestingly, in *Drosophila*, a similar expression profile was observed in the larval olfactory system, although here two pairs of conventional ORs were shown to be coexpressed in *Dm83b*$^+$ ORNs (5, 6).

**Odor Response Spectra of *An. gambiae* Larval Odorant Receptors.** Having demonstrated that a subset of *AgOrs* are expressed in larval ORNs, we used heterologous expression in *Xenopus* oocytes to

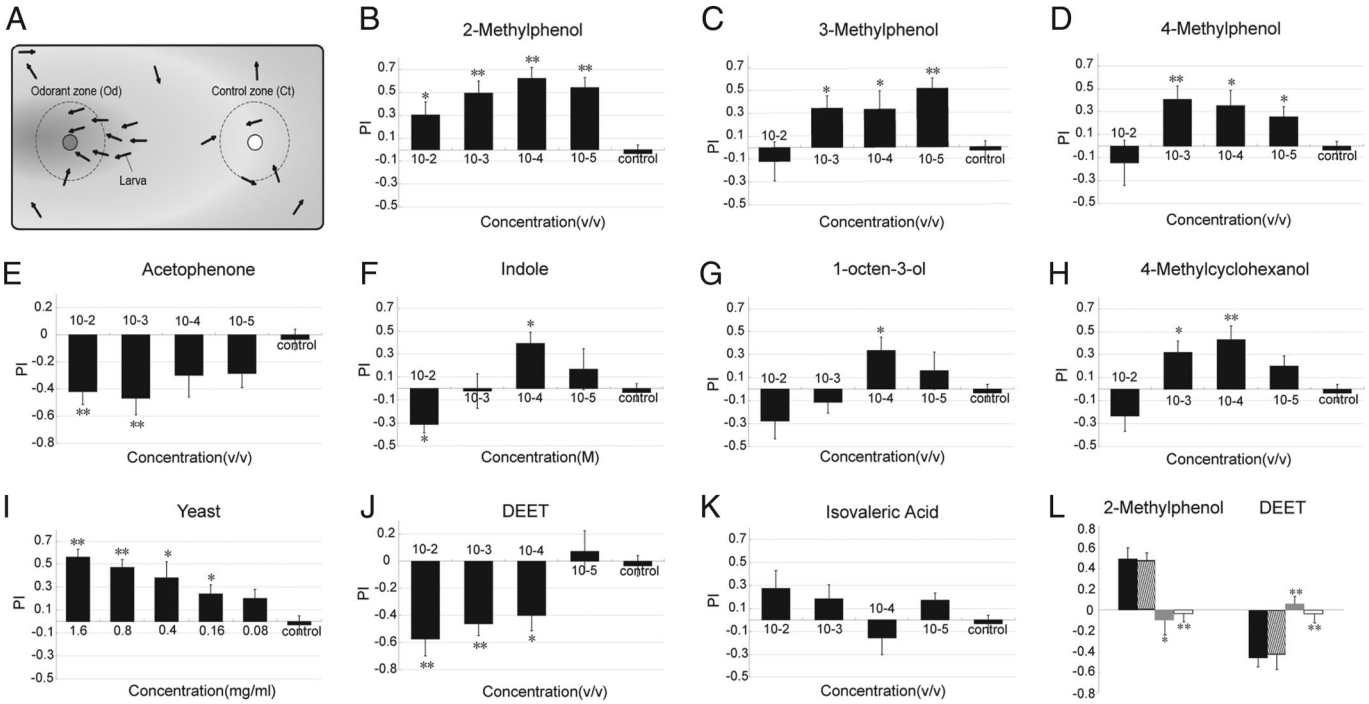


**Fig. 2.** An olfactory-based behavioral assay for mosquito larvae and odorant response profile for *An. gambiae*. (*A*) Schematic diagram of the experimental arena of the larval behavioral assay. (*B–K*) Response profiles of behaviorally active odorants: 2-methylphenol, 3-methylphenol, 4-methylphenol, acetophenone, indole, 1-octen-3-ol, 4-methylcyclohexanol, yeast, DEET, and isovaleric acid. Error bars indicate SEM for $n \geq 8$ trials. For comparisons, two-tailed unpaired student's *t* tests were performed: **, $P < 0.01$; *, $P < 0.05$. (*L*) Ablation of the larval antenna reduces olfactory responses. Behavioral responses for unablated larvae (black bars, $n = 8$); sham/maxilla ablations (cross-hatched bars, $n = 3$); antennal ablations (gray bars, $n = 3$) and no odorant/unablated control larvae (open bars, $n = 8$). Both 2-methylphenol and DEET were used at $10^{-3}$ dilutions. Error bar indicates SEM. Two-tailed unpaired student's *t* tests were performed: **, $P < 0.01$; *, $P < 0.05$ relative to unablated larvae.

examine whether these genes are functional and likely to facilitate larval olfaction. This system has been used to characterize numerous insect odorant and pheromone receptors (17–21). The test panel of 29 core chemical stimuli used for larval behavioral studies was augmented by an additional 53 compounds or odorant mixtures to enhance odorant representation across a range of chemical classes. In these functional analyses, nine larval-expressed AgORs (AgOR1, AgOR2, AgOR6, AgOR10, AgOR28, AgOR34, AgOR37, AgOR40, and AgOR48) facilitated responses to at least two odorants in the test panel (Figs. 3 and 4), whereas three (AgOR49, AgOR52, and AgOR58) failed to generate any detectable odor-induced currents in oocytes (data not shown).

Not surprisingly, the response spectrum of each individual AgOR varies. AgOR1 and AgOR34 each responded to a very narrow set of odorants, whereas AgOR10 and AgOR40 manifested much broader spectra (Figs. 3 and 4). Furthermore, the absolute response amplitude also differed significantly among AgORs. For example, the indole response current of AgOR10 was as high as ≈3000 nA, and 4-methylphenol, the strongest activator of AgOR34, only generated an ≈75-nA current (Fig. 3). It is not possible at this point to distinguish whether these effects reflect differential expression or odorant-binding affinities between individual AgORs in this system. Importantly, several of the compounds that elicited larval behavioral responses were also able to activate multiple AgORs (Fig. 4). Of these, 4-methylphenol, which evokes strong responses in behavioral assays, also produced significant currents in oocytes expressing AgOR1, AgOr2, AgOR10, AgOR34, and AgOR40. In a similar context, AgOR6, AgOR10, AgOR28, AgOR37, and AgOR40 all responded to acetophenone (Fig. 4), which evoked measurable avoidance behaviors in *An. gambiae* larvae, even at source dilutions as low as $10^{-5}$ (Fig. 2*E*). Over and above these observations, we note that most larval AgORs elicited strong responses to specific odorant groups when expressed in *Xenopus* oocytes. Each of the nine *in situ*-hybridization-positive larval AgORs responded to at least two of the aromatics tested, while AgOR1, AgOR6, AgOR10, AgOR28, AgOR37, and AgOR40 responded to a number of heterocyclic compounds. Interestingly, AgOR48 was the only larval AgOR that responded to acid, alcohols, and ketones (Fig. 4). Of the four larval-specific AgORs, two (AgOR37 and AgOR40) manifested a distinct odorant response spectra; AgOR40, however, was more of a generalist that characteristically evoked large currents and was the only larval AgOR that responded to DEET. AgOR37 appeared to be narrowly tuned to five odorants with smaller currents. Dose-response data for eight larval AgORs (Fig. S2) and AgOR28 (21) revealed $EC_{50}$ values ranging from $1.66 \times 10^{-8}$ (AgOR2 and indole) to $1.51 \times 10^{-5}$ (AgOR6 and 2-acetylthiophene).

The other two larvae-specific ORs, AgOR52 and AgOR58, showed no response to any odorants tested, suggesting that they may be tuned to a different group of odorants not included in the test panel. AgOR49, which is also expressed in adult olfactory appendages, similarly failed to yield any odorant response, suggesting that it may be tuned to undefined yet biologically significant odorants. Alternatively, the absence of oocyte responses in these instances may result from a lack of threshold levels of functional AgOR expression.

## Discussion

This work follows previous molecular studies (10) and is consistent with field and laboratory-based work in mosquitoes (reviewed in ref. 22) as well as more recent studies using *Drosophila* (5, 23). That said, it is important to appreciate that preadult fruit flies and mosquitoes reside in totally different environments. *An. gambiae* larvae inhabit small bodies of water that are often numerous,

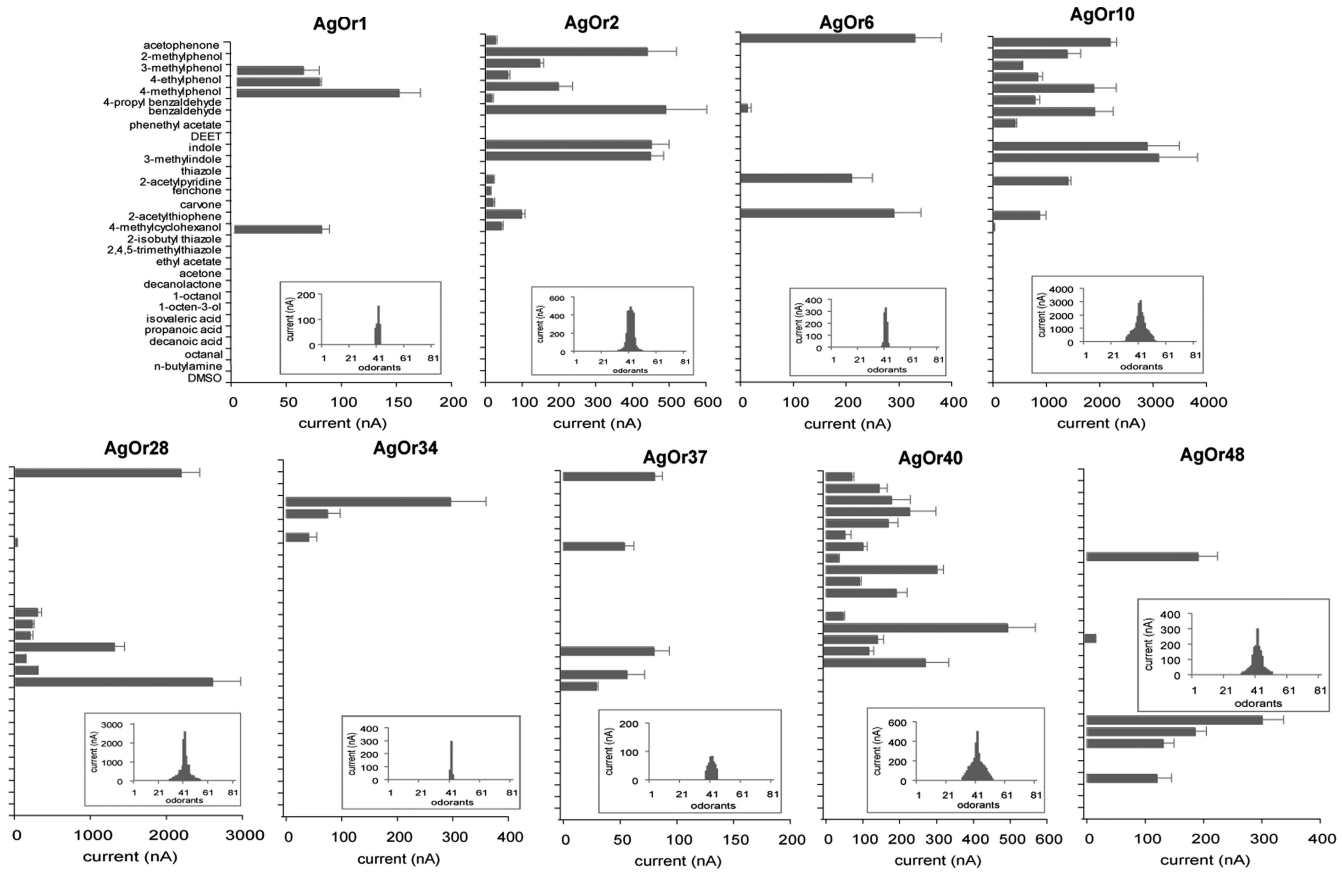


**Fig. 3.** Odor-response spectra of larval AgORs. Response is measured as induced currents, expressed in nA. Error bars indicate the SEM ($n$ = 5–8). The corresponding tuning curve for a given receptor is placed in the *Insets*. The 82 odorants are displayed along the $x$ axis, with those eliciting the strongest responses being placed near the center, and those eliciting the weakest responses placed near the edges.

scattered, sunlit, turbid, temporary, and lack consistent food resources (22), whereas *Drosophila* larvae typically exist in a terrestrial environment containing a high concentration of food. Not surprisingly, although both systems display a robust odor-coding capacity, each species has a distinct larval odor-response spectrum.

We used a simple mobility assay to identify olfactory-based responses to an odorant panel spanning multiple chemical groups and biological contexts, the majority (≈60%) of which failed to elicit any significant behavioral response. Although detailed time-lapse tracking studies are required to precisely define the nature of the odorant-induced behavioral responses of *An. gambiae* larvae we have identified, these data nevertheless provide unequivocal initial evidence of a repertoire of larval olfactory-based behaviors.

Interestingly, aromatics comprise most of the 10 odorants that were shown to be associated with significant larval responses. Of these, the positive PIs manifested by several cresol derivatives, such as 2-methylphenol, 3-methylphenol, 4-methylphenol, 4-methylcyclohexanol, and indole, over a range of concentrations, are consistent with the effects of attractants. These compounds are products of organic decay, which constitute a major food source for mosquito larvae (24). Of these, indole, 3-methylphenol, and 4-methylcyclohexanol have also been shown to evoke strong electrophysiological activity from the antennae of female adult *An. gambiae* (25). This interesting parallel between the adult and larval olfactory systems is consistent with the coexpression of several *AgOrs* in both systems as well as with the suggestion that compounds that foster larval development might also act as potential oviposition attractants for adults.

Two other compounds, acetophenone and DEET, provoked negative PIs that are consistent with potentially repulsive behaviors when tested against *An. gambiae* larvae. Acetophenone has been shown to be attractive to *D. melanogaster* larvae (5); DEET, however, is the major commercial insect repellent, although to date this has been used exclusively to target adults. 1-Octen-3-ol, which is present in the body odor of several vertebrates, including humans, and is an attractant for many insect species including *Anopheles* (26), evoked positive PIs from *An. gambiae* larvae, albeit at a single, relatively high dilution ($10^{-4}$). Although it is difficult to parse the potential biological significance of such a narrowly tuned behavioral response, it is possible that 1-octen-3-ol is normally found within the context of other compounds where it plays a synergistic role.

We examined the role of the larval antennae to define the cellular basis for these responses. Indeed, specific ablation of the larval antennae in *An. gambiae* dramatically compromised these responses, thereby validating their olfactory basis. However, larvae subjected to sham maxilla ablations and unablated controls both maintained normal response parameters (Fig. 2*L*). Consistent with our previous studies, immunohistochemistry localized *AgOR7* to define 12 putative ORN cells within the larval antennae (Fig. 1*A*). At the same time, a molecular survey of the larval antennae defined an identical number of conventional *AgOrs* that, together with *AgOr*7, are likely to be responsible for the olfactory specificity in *An. gambiae* larvae. Of these, the expression of four *AgOrs* was specifically restricted to the larval olfactory system. This is a significant overall reduction relative to the 23 Or genes that were detected in larval stages of both *D. melanogaster* (6) and *Ae. aegypti* (4), of which 10 and 15, respectively, were larval specific. Of these, apart from *AgOr7*, eight larval *AgOrs* have homologs in *Ae. aegypti*, and yet none share similarity to any *Drosophila* ORs (16). This high degree of OR conservation suggests that, although the odor space of *Ae.*

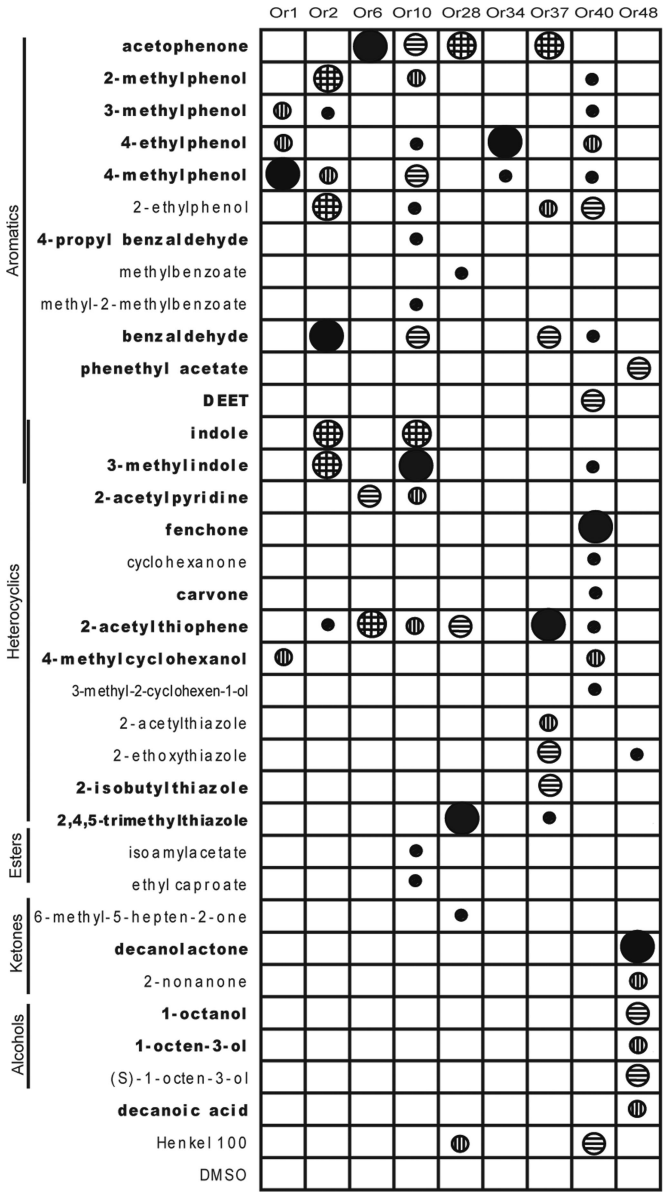


**Fig. 4.** Combinatorial coding of odors in *An. gambiae* larvae. Filled circles represent the maximal response for each AgOR. Checkered circles represent 80–99% of the maximal response of given AgOR. Horizontally striped circles represent 60–79% of the maximal response of given AgOR. Vertically striped circles represent 40–59% of the maximal response of given AgOR. Cross-hatched circles represent 20–39% of the maximal response of given AgOR. Odorants are classified into different categories according to their functional groups (aromatics, heterocyclics, esters, ketones, alcohols, and acids). The odorants highlighted in bold were also evaluated in behavioral assays.

*aegypti* larvae remains undefined, it is likely to share significant characteristics with that of *An. gambiae*, in which the overall reduction in the size of the olfactory system may reflect unique features of the larval ecology relative to other mosquito species.

Our *in situ* hybridization studies support the idea that each larval ORN stereotypically coexpresses a single conventional *AgOr* together with *AgOr7*. Although we cannot rule out that coexpression of conventional *AgOrs* ever occurs, in contrast to what has been observed for *D. melanogaster* (5, 6), this hypothesis is buttressed by several observations. These include the presence of an identical number of conventional *AgOrs* and *AgOr7*$^{+}$ ORNs as well as the absence of a single instance (over numerous double-labeling experiments) in which the transcripts for any conventional *AgOr* were detected either together with another *AgOr* or, alternatively, in more than one individual larval ORN.

Of the 82 odorants tested against nine larval AgORs, 35 evoked a response in oocytes expressing a single conventional AgOR. Although the inherently limited odorant panel precludes any assumption that this represents the complete response spectrum, it is nevertheless sufficiently broad to suggest that these data provide a comprehensive survey. Although several AgORs—AgOR2, AgOR10, AgOR28, and AgOR40—manifested relatively broad tuning responses and AgOR1, AgOR6, and AgOR34 exhibited a more narrowly tuned response, there was an overall bias toward compounds with aromatic and heterocyclic functional groups. Indeed, of the nine AgORs that functioned in *Xenopus* oocytes, only AgOR48, which is tuned to a modest number of odorant ligands, displayed any significant responses outside the aromatic/heterocyclic range. Moreover, in keeping with the paradigms established in vertebrate (27) and in both larval (6) and adult (28) *Drosophila* systems, several odorants elicited responses from multiple AgORs (Fig. S2). In a similar manner, this suggests that there is a functional redundancy insofar as AgOR tuning and that a combinatorial coding mechanism acts to encode odorant information in *An. gambiae* larvae. The most striking element of this analysis was the relatively large number of odorants that activated a very narrow range of the larval AgOR repertoire. Indeed, just over half of the 35 active odorants in this survey elicited responses from a single larval AgOR; if one considers odorants that activate up to two larval AgORs, this rises to >65%. This is consistent with similar observations for combinatorial odor coding in the larval olfactory system of *Drosophila* (6) and may reflect an implicit restriction in how the response spectrum is maintained in a significantly less complex neuronal system.

The link between peripheral olfactory sensitivity and larval behavioral output is obvious, although not straightforward. In this context, we can easily rationalize the effects of odorant stimuli, such as acetophenone and 2-, 3-, or 4-methylphenol, which stimulated multiple AgORs and also evoked robust larval behaviors. Similarly, we never observed a behaviorally active stimulus that failed to activate at least one functionally characterized larval AgOR. One of the most striking examples is the behavioral and AgOR40-expressing oocyte responses to the adult insect repellent DEET (Figs. 2*J* and 4), which, based on the larval specific expression of *AgOr40*, may indicate the presence of additional DEET-sensitive AgORs in the adult. An alternative suggestion is that DEET may not act as a true behavioral repellent but rather by inhibiting the attraction to another compound, perhaps through antagonistic mechanisms at the molecular level (15, 29).

Several compounds, such as 2-acetylthiophene, benzaldehyde, and 3-methyl indole, strongly activated multiple AgORs in *Xenopus* oocyte recordings, yet did not evoke significant behavioral responses from *An. gambiae* larvae within the context of our assays. This may result from an inherent limitation of our behavioral paradigm or it might be that sensory input at a molecular level is necessary but may not always be sufficient to generate behavioral responses. This is not unique to mosquitoes, as ethyl acetate activates three ORs expressed in *Drosophila* larvae (6, 28), despite the fact that wild-type *Drosophila* larvae are largely indifferent to ethyl acetate in a chemotaxis assay (5).

Aqueous larval habitats inherently represent a confined and therefore more easily targeted venue for mosquito control strategies (2). In addition to addressing the underlying basis for olfactory coding in insects, these studies shed light on the mechanistic elements of the larval olfactory system in Anopheles that may facilitate the development of novel approaches targeting larval feeding and other behaviors to potentially enhance the effectiveness of current vector control strategies.

## Methods

**Mosquito Rearing.** *An. gambiae sensu stricto*, originated from Suakoko, Liberia, and was reared as described in ref. 30. For stock propagation, 4- to 5-day-old female mosquitoes were blood-fed for 30–45 min on anesthetized mice, following the guidelines set by Vanderbilt Institutional Animal Care and Use Committee.

**Scanning Electron Microscopy.** Larva heads were hand dissected and prepared and visualized under a Hitachi S-4200 scanning electron microscope (SEM) as described in ref. 31.

**Reverse Transcriptase PCR.** Antennae were hand-dissected from *An. gambiae* third-instar larvae. RNA extraction, cDNA synthesis and PCRs were performed as described in ref. 32. PCR amplification products were run on a 1.5% agarose gel and verified by DNA sequencing. For each individual *AgOr*, three independent PCR trials were performed along with appropriate controls.

***In Situ* Hybridization.** Heads were hand-dissected from *An. gambiae* third-instar larvae, embedded in paraffin, and sectioned at 8 $\mu$m on a sliding microtome (HM340E; Microm). Fluorescence *in situ* hybridization and immunohistochemistry were performed as described in ref. 21.

**Receptor Expression in *Xenopus* Oocytes and Two-Electrode Voltage-Clamp Electrophysiological Recording.** Full-length coding sequences of larval *AgOrs* were PCR amplified from third-instar larval antennae cDNA. Whole-cell currents were recorded from the *Xenopus* oocytes injected with corresponding cRNAs by using a two-electrode voltage clamp as described in ref. 21.

**Larval Behavior Assay and Data Analysis.** One hundred *An. gambiae* second or third instar larva were picked and washed carefully. Washed larva were kept in 27°C distilled water and starved for 2 h. Odorant stocks were made by dissolving a specific amount of the odorants in preheated 2% NuSieve, GTG low-melting temperature agarose (Cambrex Bio Science). The assay was performed in a 38.1 $\times$ 25.4 $\times$ 5.08-cm Pyrex dish containing 500 ml of 27°C distilled water. A test zone and control zone were determined and outlined. The larva were released in the center of the dish and allowed to swim freely for 1 h. The odorant/control stock was inserted into a mesh ring and then placed in the center of the zone area accordingly. Real-time images were acquired every 30 s over a 22-min assay. The number of larvae in both test and control zones were counted at all time points. In all cases, we calculated a performance index (33) at a discrete15-min time-point as follows: $PI = (\#_{odorant} - \#_{control})/(\#_{odorant} + \#_{control})$, where the $\#_{odorant}$ indicates the number of larvae in the test zone and the $\#_{control}$ indicates the number in the control zone. Respective PI values were compared with each other and analyzed for statistical significance by using unpaired, two-tailed student's *t* tests with Prism software (GraphPad).

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