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Olfactory learning and chemical ecology of olfaction in disease vector mosquitoes: A life history perspective

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Abstract

Mosquitoes transmit many debilitating diseases including malaria, dengue and Zika. Odors mediate behaviors that directly impact disease transmission (blood-feeding) as well as life history events that contribute to mosquito survival and fitness (mating and oviposition, nectar foraging, larval foraging and predator avoidance). In addition to innate olfaction-mediated behaviors, mosquitoes rely on olfactory experience throughout their life to inform advantageous choices in many of these important behaviors. Previous reviews have addressed either the chemical ecology of mosquitoes, or olfactory-driven behaviors including host-feeding or oviposition. Adding to this literature, we use a holistic life history perspective to integrate and compare innate and learned olfactory behavior at various stages of mosquito development.

Introduction

Many mosquito control strategies target the mosquito olfactory system at both the larval and adult stages. Larval control strategies can target chemosensory mediated behaviors with techniques such as predator introduction and ingestible microbial larvicides [1]. Predators emit chemosensory cues¹ which larvae can sense and avoid [2], and larvae may use chemosensory cues to recognize ingestible larvicides as food. Although microbial larvicides are highly effective [1, 3] and harmless to nontarget organisms [1], increased larval resistance [3] highlights the possible need for additional control options. A better understanding of larval olfactory deterrents may help identify new microbial larvicides. At the adult stage, mosquito control methods include olfactory attractants (e.g. carbon dioxide, 1-octen-3-ol, Attractive Toxic Sugar Baits [4-8]) and repellents (e.g. DEET, picaridin [9]). Olfaction thus represents a major target in effective control of mosquito populations.

Here we discuss mosquito olfactory experience during both the larval and adult stage. We begin by addressing the behavioral role of olfaction throughout the mosquito life cycle.

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¹In many papers describing mosquito chemosensory ecology, particularly those involving larvae, the chemical compounds are not identified and the mechanism of mosquito behavior is not examined directly. Therefore, we cannot preclude the possibility that some semiochemicals may be processed by other chemosensory systems (eg, gustatory system). Throughout this review we use the term “chemosensory” to describe findings in which this is particularly unclear.

These behaviors include larval foraging, larval predator avoidance, adult nectar foraging, host selection, mating, and oviposition (Figure 1) [10]. Associative and non-associative olfactory learning can also increase individual fitness by refining behavioral choices throughout a mosquito's life [10]. Next we give a brief overview of the comparative olfactory neuroanatomy of mosquito larvae and adults. Finally we examine promising areas of future research in the field of mosquito chemical ecology, and argue that the similar neurobiological processes mediating experience-dependent behavioral plasticity at the adult and larval stages may provide suitable targets for mosquito control.

Olfactory Learning and Behavior in Mosquito Larvae

Unlike adults, many disease vector mosquito larvae inhabit small aquatic environments including flower vases and water pots [13]. Due to spatial restrictions in these habitats, larvae may be particularly vulnerable to predation and lack of food. Chemosensory indicators of these dangers are thus ecologically relevant to both adult and larval mosquitoes. It is advantageous for gravid adult females to recognize and choose oviposition sites with ample food and few predators [13,14,15] (See detailed discussion in “Olfactory cues involved in mating and oviposition”). In addition, there is likely a fitness advantage to larvae using chemosensory cues to avoid predator kairomones [2] and seek out olfactory indicators of food [13,16]. Deorphanization of olfactory receptors (ORs) expressed in both larvae and adult females may help identify key olfactory signals shared in oviposition and larval fitness.

Spatial restriction may also threaten larvae with extreme changes in the physical-chemical environment (temperature, acidity, plant-derived compounds, and salinity), as smaller volumes of water are susceptible to rapid temperature change and chemical pollutants. In fact, *Aedes aegypti* larvae exhibit many physiological adaptations to these conditions: They can survive freezing for 8 hours, eclose when reared in pH ranging from 4.4 to 9.5, or in water containing 0.9% salt [13]. Many insects learn and respond to cues associated with the physicochemical environment [17], making these stimuli suitable candidates to test in aversive learning assays.

Larval olfactory responses to food resources

Larval attraction to food is robust and mediated by olfaction [16,18]. The three major disease vector mosquito genera (*Aedes*, *Anopheles*, *Culex*) are larval detritivores, and products of organic decay such as cresol derivatives (indole, 2-methylphenol, 4-methylcyclohexanol) are innately attractive to *Anopheles gambiae* larvae [18]. In *Ae. aegypti*, the axon guidance gene *semaphorin-1a* has been identified as an important component of larval olfactory development and attraction to yeast [19]. Nonetheless, microbes produce more than 1000 different volatile compounds [20], and it is unclear which semiochemicals are involved in larval attraction to food. In adult *Ae. aegypti*, many bacterial kairomones affect oviposition in a concentration-dependent manner: Some bacterial species deter oviposition while others stimulate egg-laying, and many kairomone isolates (nonanoic acid, tetradecanoic acid, methyl tetradecanoate) stimulate oviposition only at a narrow concentration range [21]. It remains unknown whether larvae also use this large dimensional chemical space [20] to modulate their behavior, or can be learned by the larvae in an

associative manner. However, the sensitivity of ovipositing females to bacterial kairomones [21] suggests that the composition of the microbial community is important to larval fitness. Further, several microbes (*Bacillus sphaericus*, *Bacillus thuringiensis* var. *israelensis*) cause larval death within 48 hours [1]. As such, chemosensory indicators of dangerous bacterial communities may be innately aversive to larvae, or readily learned as aversive when encountered alongside toxic bacterial byproducts.

Olfactory experience in larval predator avoidance

An important fitness-related effect is for mosquito larvae to avoid predation in small, spatially restrictive, pools [2,13], and indeed, mosquito larvae have been shown to display species-specific avoidance of chemosensory cues from predators [2]. For example, *Culex pipiens* larvae can detect chemosensory cues from *Notonecta undulata*, a natural predator of this larval species [2]. *Cx. pipiens* show significant refuge-seeking behavior and movement reduction in water containing *N. undulata* chemical cues [2]. However, *Ae. aegypti* larvae (a species with no evolutionary history of *Notonecta* predation) do not respond to *N. undulata* odor [2], suggesting that there may be species-specific olfactory tuning to predator cues. Given the extremely limited number of larval ORs (see detailed discussion in “The Neurophysiological Bases of Mosquito Olfactory Learning”), it is possible that larvae simply do not express ORs tuned to ecologically irrelevant predators. Comparative studies examining inter-species differences in chemosensory responses to predator-related cues could reveal whether these behavioral differences are caused by differences in sensory detection.

In addition to innate predator avoidance [2], mosquito larvae can learn new associations between predator odors and perceived danger [22]. In *Cx. restuans*, larvae exhibit movement reduction in the presence of injured conspecific alarm cues [22]. Although naive *Cx. restuans* larvae do not change their behavior in the presence of salamander odor, larvae conditioned with salamander odor and crushed conspecifics demonstrated significant movement reduction in salamander odor alone [22]. This study did not characterize the chemical composition of salamander odor, but *Notonecta maculata* – another predator of larval mosquitoes – release cuticular hydrocarbons (*n*-heneicosane, *n*-tricosane) that repel ovipositing *Culiseta longiareoleta* [23]. Larvae may use similar cues, but the mechanism of chemosensory predator detection is still unknown. Further, the *An. gambiae* larval olfactory system includes both ORs and ionotropic receptors [16], raising the possibility that multiple chemosensory channels may be involved in predator detection.

Olfactory Learning and Behavior in Adult Disease Vector Mosquitoes

Olfactory cues involved in food-seeking behaviors

Sugar meal: While female disease-vector mosquitoes require a blood-meal to produce and mature their oocytes, both females and males also seek carbohydrates (sugars) as a source of energy to sustain their metabolism and behavior [13]. Energetically, sugar and blood are interchangeable [24], and sugars represent the sole food for males and, in some non-blood-feeding species, for both males and females (e.g. *Toxorhynchites* spp. mosquitoes). Mosquitoes find carbohydrates by visiting plants that provide floral and extrafloral nectar,

ripe fruits, honeydew, and tree sap [24] which also contain other nutrients such as amino acids. A wide range of volatile compounds are emitted by these sugar sources, including aldehydes, alcohols, ketones, phenols and terpenes [25], which are detected by the mosquito olfactory system (Figure 2). Despite their potential importance, the chemical identities and blends that mediate responses by mosquitoes to nectar resources remains largely uncharacterized.

Combined fieldwork and laboratory assays have shown that male *An. gambiae* mosquitoes exhibit a preference for specific plant species (e.g. *Mangifera indica* (Anacardiaceae), *Thevetia nerifolia* (Apocynaceae), and *Doloxia regia*, *Senna siamea*, and *Cassia sieberiana* (all Fabaceae)) [35]. Females are equally attracted to specific plant odor bouquets and to synthetic blends based on the plants' scent composition [36]. Interestingly, several plant species that attract *An. gambiae* produce both floral and extrafloral nectar, and all emit terpenoids [37], including monoterpenes such as α -pinene, D-limonene and β -myrcene, that are common constituents of essential oils described in the literature as presenting mosquito repellent activity [38].

This olfactory-mediated attraction to plants is not limited to *Anopheles* mosquitoes. Both male and female *Cx. pipiens* are attracted to plant extract and artificial chemical blends [39,40]. In addition, a successful nectar meal can constitute a positive reinforcement for mosquitoes and modulate their behavior based on past experience [41]. For example, the attraction to floral scent increased for both male and female *Cx. pipiens pipiens* when the floral scent was paired with a 5% sucrose solution [41]. In *Cx. quinquefasciatus* both males and females could learn the association between odors and a sugar meal [42]. It is worth noting that not only do mosquitoes respond to plant scents [35,36], but some species of mosquitoes (e.g. *Ae. (Oc.) communis*, *Ae. (Oc.) canadensis*) also pollinate these plants when nectar feeding [43]. Finally, sugar-feeding and blood-feeding are not completely independent. Blood and sugar feeding activities are often observed in the same activity period and have been described as antagonistic and mutually exclusive [24]. Although sugar intake increases the mosquito lifespan, biting rates are usually reduced when sugar is available [44], suggesting a potential epidemiological impact of the blood-feeding versus sugar-feeding conflict as the absence of sugar sources would increase vectorial capacity [44]. However, whether sugar feeding experience, by increasing attraction to floral scents, affects host biting frequency is still to be determined.

Blood-meal: Female mosquitoes have been shown to exhibit preferences for a given host species (including humans for anthropophilic mosquitoes such as *An. gambiae* [45] and *Ae. aegypti* [13]), yet other species (e.g. *Cx. nigripalpus*, [46]) change their host preference in response to seasonal variations in host availability or changes in host defensive behaviors such as antiparasitic and grooming behaviors [47]. In this context, olfactory signals may be particularly valuable as they allow mosquitoes to identify preferred hosts from a safe distance. Furthermore, mosquitoes can learn from previous encounters with host olfactory cues and use this learned information to select hosts that are the least defensive and easiest to feed on [48-50]. By modulating responses to host-related olfactory cues such as L-(+)-lactic acid and 1-octen-3-ol [51-53], as well as impacting the efficiency of popular insect repellents such as DEET [53], individual experience certainly plays an important role in

mosquito host choice and preference. As learning abilities might affect mosquitoes biting behavior patterns amongst host populations, and consequently the rate of parasite transmission, it is thus important to consider them in epidemiological models and to develop efficient control strategies [49, 53-56].

Olfactory cues involved in mating and oviposition

Chemosensory cues play an important role in mosquito social interactions and mating behavior, and these responses can be modulated by the prior experience and physiological state of the haematophagous insect. For instance, evidence for aggregation pheromones has been found in *Cs. inornata* [57] and more recently in *Ae. aegypti* mosquitoes [58]. Interestingly, when the odors of male mosquitoes and the odor of a potential host (i.e. a rat) were presented together, *Ae. aegypti* females showed a typical flight response towards the male odors but did not respond to the host signal [59]. This highlights how the reproductive state can modulate the response to host odors and affect host-seeking behaviors, although the volatiles mediating this attraction have yet to be identified. Several other studies support the hypothesis of the presence of a contact sex pheromone that could be used for sex and conspecific recognition [60], but the chemical composition of these substances has yet to be determined, and whether these behaviors can be modulated by previous experience remains an open question in mosquitoes. However, in other haematophagous insects such as the gregarious species *Triatoma infestans*, the attraction to aggregation pheromones is modulated by previous experience [61]. Their innate attraction to this latter could even be switched to a learned aversion after associative conditioning [61].

Mated female mosquitoes use various signals (visual, humidity, olfactory etc.) to locate a place to oviposit, and evidence exists that this behavior can be modulated by the mosquitoes prior experience. For example, *An. coluzzii* can detect deterrent chemicals associated with overcrowded pools of water, and avoid these specific areas as they would not be suitable for the optimal development of progeny [62]. In *Cs. longiareolata*, females are able to detect the presence and density of predators such as *Notonecta* and avoid laying eggs in these specific water pools [63]. Similarly, *Culex* mosquitoes avoid ovipositing in water that previously contained larval predators, suggesting the use of predator-released kairomones [64]. While some *Notonecta* kairomones have been identified (*n*-heneicosane, *n*-tricosane) [23], less is known about similar cues from other predator species. In malaria vectors, it has been shown that olfactory cues not only from conspecifics [65,66] and microorganisms [67] but also from the soil (e.g. the sesquiterpene cedrol) [68] or the surrounding environment [69,70] are used by gravid females for oviposition site selection. In Zika, dengue and yellow fever vectors, bacteria present in the water have been shown to increase the oviposition rate [71] as well as the presence of conspecifics [72-74] and chemicals resulting from decaying vegetation (e.g. *p*-cresol) [75]. Like nectar- and host-seeking, oviposition site preference can also be modulated by individual experience in *Cx. quinquefasciatus* [76] and *Ae. aegypti* [77], although the cues mediating these responses, and the nature of the learned response (i.e. associative or non-associative) remains unknown. It thus seems that, in addition to innate olfactory behaviors, individual experience plays a role in various steps of the adult mosquitoes' life [78,79].

The Neurophysiological Bases of Mosquito Olfactory Learning

Several articles describe the beginning stages of olfactory information processing in adult mosquitoes [80-82], but detailed understanding of the neural substrates of olfactory plasticity remains an open question. In adults, information flows from ORs and CO₂-sensitive receptors located on the antennae and the palps, through the antennal lobes (AL), to higher order brain areas such as the lateral horns and mushroom bodies, thought to be involved in learning and memory (Figure 2A,B). Compared to the adults, the larval olfactory neuroanatomy is numerically simpler. Adult female *Ae. aegypti* have ~3800 putative antennal olfactory receptor neurons (ORNs) [29], while larvae of the same species only have ~24 [27]. Interestingly, there are 21 putative OR genes expressed in *Ae. aegypti* larval antennae [30]² – far more than the 12~13 ORNs found in each antenna [27]. This comparatively large number of expressed OR genes is unusual for the 1OR:1ORN relationship typical of insects [30]. *Ae. aegypti* larvae might express multiple ORs on the antennal gustatory receptor neurons, or express different ORs based on developmental stage, sex, or nutritional state. Electrophysiology and deorphanization studies have thus far focused primarily on adults [80,83]. Similar work would be of interest in larvae, as 14 of 21 *Ae. aegypti* larval ORs are larvae-specific [30], as well as 4 of 12 in *An. gambiae* larvae [18].

While understanding how odor information is processed in higher-order brain centers remains an open question in both adults and larvae, a handful of neuroanatomical studies have been conducted in adult mosquitoes [81,84]. While the structure and function of larval mushroom bodies (Figure 2C) is not well studied, our work has shown there is remarkable similarity in the physical neuroanatomy of the mushroom body between the larval and adult stages (Figure 2C). In *Drosophila melanogaster* [85] and *Ae. aegypti* [19] the larval olfactory system has many fewer neurons than adults, but exhibits strong similarities in overall architecture. Although more work is necessary to characterize comparative adult and larval olfactory neuroanatomy in other mosquito species, the relatively simple larval system may be a useful model for investigating key aspects of mosquito olfaction conserved across life stages.

What are possible neural mechanisms that permit behavioral flexibility by larvae and adult mosquitoes, and how is the odor information coupled with the appetitive “reward”, or aversive “punishment”, to form the learned association? Work in many insects, including our own work with *Ae. aegypti* mosquitoes, has shown the importance of neurons that release amine neuromodulators (eg, serotonin, dopamine or octopamine), and their cognate receptors, for olfactory learning [17,50,86]. For example, a large serotonin-immunoreactive neuron innervates all glomeruli in the *Ae. aegypti* AL, and sends axon branches to the mushroom body calyces, the central complex – both brain regions thought to operate in memory-related behaviors –, the lateral horn, and receptors are expressed in these brain regions as well as on the mosquito antennae and maxillary palps [33,87]. Similarly, large dopamine-immunoreactive neurons innervate the AL and superior medial protocerebrum, and dopamine receptors are expressed at high levels in these brain regions [50]. Pharmacological interventions and genetic manipulations of dopamine receptors selectively

²not including two pseudogenes and the obligate olfactory coreceptor Orco.

suppress olfactory learning in *Ae. aegypti*, suggesting the dopamine pathway is critical for learning and memory [50]. Thus, these amines may provide the reinforcement system to increase olfactory responses from the AL and mediate the formation of memory in the mushroom bodies [50,86]. Work from diverse insects, including *Drosophila* and honeybees, supports this general hypothesis: when stimulated by the unconditioned stimulus (US) neuromodulatory neurons are activated leading to neurotransmitter release in the AL and mushroom bodies. The coincident input from the AL (via the odor stimulus, or conditioned stimulus [CS]) with dopamine release causes synaptic growth in mushroom body neurons and memory formation.

How neuromodulators like dopamine influence olfactory circuits and odor representations in mosquitoes remains an open question. In specific brain regions, innervation by large neuromodulatory neurons can be global, or confined to specific sub-regions and possibly sub-circuits. For instance, in the *Ae. aegypti* AL the serotonin-immunoreactive neuron has a similar innervation pattern across glomeruli [87], whereas the dopamine-immunoreactive neuron selectively innervates some glomeruli more than others (eg, the MD glomeruli, which respond to host volatiles) [50]. Serotonin and tyrosine hydroxylase, a dopamine precursor, are also expressed in the *Ae. aegypti* larval antennal lobe and lateral protocerebrum (Personal communication, Gabriella H. Wolff), possibly suggesting conserved functions between life-history phases. While the relative function of dopamine, serotonin and octopamine is unclear, partly due to their overlapping and cooperative interactions [88], nonetheless, studies have shown they are critical for diverse processes, including circadian activity state, arousal, and learning [17,86], suggesting that these neuromodulators serve as “control knobs” for specific olfactory channels, thereby allowing certain odors to be processed under different environmental and physiological conditions.

Conclusions and Prospects for Future Research

We have provided an overview of studies investigating the role of olfaction during the mosquito life and its role in ecologically and epidemiologically important behaviors. Individual experience, historically neglected in epidemiological models and control strategies, plays a role in the majority of olfactory behaviors of both larvae and adults. Some olfactory cues, such as larval predator odors and larval food odors, are used by both adults and larvae. These odors also play a critical role for adult female mosquitoes when choosing an oviposition site. However, how these odors are used by mosquitoes in an experience-dependent manner remains an open question. Given the importance of olfactory learning throughout mosquito life history and its interaction with commonly used pesticides [53], a better understanding of the mechanism and limitations of learning may help improve the application of existing olfactory control techniques. Thus far, behavioral experiments, single-sensillum electrophysiological recordings, and ORN staining studies have contributed to a better understanding of neural mechanisms behind these behaviors [80-83]. However, several urgent questions remain in the field of mosquito olfaction.

First, there is a dearth of information on the neuroanatomy of higher order brain structures in mosquito larvae. Due to the numerical simplicity of larval neuroanatomy, mosquito larvae are an attractive model for understanding the neural encoding of mosquito olfactory

behavior. Analyzing the physical structure and interconnectivity of higher order larval brain areas is a key step in studying functional changes that occur during mosquito learning and memory, as well as providing the basis for comparing learning and memory between the different life-history stages.

Secondly, the breadth of chemosensory ligands and their cognate receptors that mediate mosquito behavior is still unknown, particularly in larvae. Although *Xenopus* receptor expression studies have characterized the tuning properties of many *An. gambiae* larval ORs [18], similar information is lacking for other species. Importantly, the mosquito olfactory subgenome exhibits significant divergence between genera [30,89]. A cross-species study of the full range of behaviorally relevant semiochemicals is crucial to understanding adult and larval chemical ecology, de-orphanizing olfactory receptors, identifying associated neurobiological functions, and inter-species differences in mosquito behavior.

In addition, the mechanism of discrimination between complex odors is not well understood. As discussed, mosquitoes respond to olfactory information from a wide variety of sources including plants, animal hosts, conspecifics and oviposition sites. These odors often consist of several hundred different chemical compounds [90,91], many of which are shared amongst unrelated odor sources [83,92]. Decoding the key compounds in mosquito mixture processing may help explain some of the differences in intra- and inter-species host attractiveness.

Last, determining how neuromodulatory systems influence the processing of olfactory information remains an important gap in mosquitoes. Amines like dopamine – critical for aversive and appetitive learning – may influence local and regional brain areas, but such determinations remain untested. An outstanding question in olfactory neurobiology is thus identifying how aminergic neuromodulators selectively modulate specific olfactory circuits, and how that modulation transforms information processing in downstream areas of the brain. Given the importance of learning and associated neuromodulatory systems in diverse life-history processes, future studies on these topics could facilitate our understanding of mosquito-host interactions and potentially identify genetic targets for vector control.

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Highlights

1. Many olfactory cues affect innate and experienced behaviors throughout the mosquito life cycle.
2. Larval environment odors are ecologically relevant to both larvae and gravid adults.
3. Olfactory experience mediates important behaviors like feeding and oviposition.
4. The olfactory channels and neural substrates involved in experience-dependent behaviors provide potential targets for insect vector control.

Figure 1.

Known physiological and external influences on mosquito olfactory behavior [10]. Behaviors marked in yellow have been shown to be mediated by olfactory experience. These behaviors can also be modulated by physiological factors (blue), which are reviewed elsewhere [11,12].

Figure 2.

(A) Neuroanatomy of the *Ae. aegypti* olfactory system. Top: larva [13]. Bottom: adult female [13,26]. AN: antennae, GB: germinal bud of adult antennae, ON: olfactory nerve, AL: antennal lobe, CO₂: CO₂-sensitive receptor neurons and afferents, B: other regions of the brain.

(B) Distribution of chemosensory sensilla in the *Ae. aegypti* antennae and maxillary palps. B1: larval antennae [27], B2: adult male antennae [13,28], B3: adult female antennae [13,29], B4: adult male maxillary palps and proboscis [13,30-32], B5: adult female maxillary palps and proboscis [13,26,32,33]. ORN: olfactory receptor neurons. GRN: gustatory receptor neurons, including CO₂-sensitive receptor neurons.

(C) Confocal scan of the mushroom bodies of *Ae. aegypti* L4 stage larvae. Anti-DC0 (magenta, used here at a concentration of 1:250) recognizes the catalytic subunit of cAMP dependent protein kinase A in all arthropods investigated thus far [34]. This anti-DC0 was a generous gift from Dr. Daniel Kalderon, Columbia University. A monoclonal antiserum against α-tubulin (cyan, 12G10) was also used at a 1:100 concentration (developed by Drs. J. Frankel and E. M. Nelsen at NICHD, obtained from the Developmental Studies Hybridoma Bank).