Chemical Senses, 2021, Vol 46, 1–24
doi:10.1093/chemse/bjab021
Review Article
Advance Access publication 22 April 2021

OXFORD

Review Article

Olfaction in *Anopheles* mosquitoes

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Editorial Decision 12 April 2021.

Abstract

As vectors of disease, mosquitoes are a global threat to human health. The *Anopheles* mosquito is the deadliest mosquito species as the insect vector of the malaria-causing parasite, which kills hundreds of thousands every year. These mosquitoes are reliant on their sense of smell (olfaction) to guide most of their behaviors, and a better understanding of *Anopheles* olfaction identifies opportunities for reducing the spread of malaria. This review takes a detailed look at *Anopheles* olfaction. We explore a range of topics from chemosensory receptors, olfactory neurons, and sensory appendages to behaviors guided by olfaction (including host-seeking, foraging, oviposition, and mating), to vector management strategies that target mosquito olfaction. We identify many research areas that remain to be addressed.

Key words: chemosensory receptors, host-seeking, malaria, olfactory neurons, sensory appendages, vector management

Introduction

There are ~3600 recognized species of mosquito (Diptera: Culicidae) belonging to 3 subfamilies: Anophelinae, Culicinae, and Toxorynchtinae (Harbach 2007, 2013) found on all continents except Antarctica. Despite popular impressions, not all mosquitoes are hematophagous (i.e., feed on blood). For example, adult Toxorynchtinae mosquitoes, which are the largest of all mosquitoes, feed only on nectar. Adult females of some Anophelinae (*Anopheles*) and Culicinae (e.g., *Aedes* and *Culex*) mosquitoes however do require a blood meal to complete egg development. Hematophagy in arthropods evolved independently multiple times from pre-existing associations with vertebrates or plants (Grimaldi and Engel 2005; Lehane 2005). What probably started as accidental feeding on vertebrates, over time led to morphological (e.g., mouthparts), physiological (e.g., salivary secretions, blood digestions), and behavioral (e.g., host preferences) adaptations to feed on blood (Graça-Souza et al. 2006; Mans 2011; Krenn and Aspöck 2012; Arcà and Ribeiro 2018).

A mosquito blood meal can come from a variety of vertebrate (e.g., birds, frogs, mammals, snakes) and occasionally invertebrate (e.g., earthworms, leaches) hosts (Clements 1999; Reeves et al. 2018; Wolff and Riffell 2018). Although some degree of plasticity in host preference exists, females of each blood-feeding mosquito species usually specialize on one host type. For some mosquitoes, the preferred hosts are humans. This requirement for blood and the close association with and preference for human hosts makes these mosquitoes capable of vectoring disease-causing pathogens. Several viruses and parasites, which affect millions of people globally each year, are vectored specifically by mosquitoes. For example, *Culex* mosquitoes vector West Nile virus, whereas *Aedes* mosquitoes vector viruses, which cause chikungunya, dengue fever, yellow fever, and Zika (Farajollahi et al. 2011; Lee et al. 2018; Weaver et al. 2018; Huang et al. 2019).

The deadliest of all anthropophilic (human preferring) mosquitoes are those of the genus *Anopheles*, capable of vectoring the malaria-causing *Plasmodium* parasite, which kills over 400 000 people each year (WHO 2019). Although different species of *Anopheles* might opportunistically feed on human and nonhuman hosts (Dekker et al. 2002; Bakker et al. 2020), malaria vectors

such as *Anopheles gambiae* are predominantly anthropophilic and are highly attracted to and specialized to blood-feed on humans (Braks et al. 1997; Costantini et al. 1998; Pates et al. 2001). Species of *Anopheles* mosquitoes are found on every continent (except Antarctica), in over 100 countries. Their global distribution is limited by temperature, with declining survival below 15°C and above 35°C (Lyons et al. 2012, 2013).

Blood-seeking female mosquitoes must detect and interpret information about their environment, and thus rely on different sensory modalities and a finely tuned sensory system. Olfaction is an important sensory modality for adult female mosquitoes as it is involved in foraging, host-searching, and oviposition site selection. Most importantly, it is primarily through olfaction that female mosquitoes locate and recognize a specific host for blood-feeding, and potentially transmit diseases. Therefore, targeting mosquito olfaction can reduce the number of infectious bites and presents opportunities for effective interventions.

Substantial advances have been made over the past decade in understanding the mosquito olfactory system. Olfactory cues are detected by receptors on sensory appendages, which activate olfactory sensory neurons (OSNs) to carry the signals to the brain. Interpretation of those signals by the brain leads to olfactory guided behaviors, including host-seeking. Although it is often assumed that all mosquitoes rely on the same host-seeking mechanism (i.e., the molecular basis underlying these behaviors), there are probably marked differences among major subfamilies, which remain unexplored. Research on *Anopheles* mosquitoes has traditionally focused on vector competence and control to address the public health challenges associated with malaria transmission and eradication (Ferguson 2018; Greenwood et al. 2008). Combining current control efforts with a deeper understanding of vector biology, behavior, and the mechanisms underlying them has the potential to guide novel interventions (Shaw and Catteruccia 2019).

In this review, we synthesize the current state of knowledge and discuss recent advances in our understanding of olfaction in *Anopheles* mosquitoes from signal perception and processing of olfactory cues at the neuronal level to elicited behaviors at the organismal level. First, we describe the olfactory system in *Anopheles* mosquitoes: olfactory receptors, peripheral olfactory appendages, and higher olfactory centers (Figures 1 and 2; Table 1). Then, we discuss the role of olfaction in adults focusing on females, from foraging and mating, to host-searching, blood-feeding, and oviposition (Figure 3). Finally, we outline the role mosquito olfaction plays in malaria transmission (Figure 4) and highlight integrated vector management strategies involving olfaction. Our goal is to bring attention to recent developments and discoveries and highlight promising avenues for future work in *Anopheles* olfaction.

Olfactory system in *Anopheles*

There are 3 peripheral olfactory appendages in *Anopheles* mosquitoes: the antennae, the maxillary palps, and the labella on the proboscis (Figure 1). All share some basic features also found in other insects: the appendages are covered in sensory hairs called sensilla; each sensillum is innervated by the dendrites of olfactory neurons expressing various combinations of olfactory receptors (Figure 2; see Olfactory receptors and coreceptors). When odorant molecules bind to these receptors, the neurons fire, sending an olfactory signal via their axons to the primary processing center(s) in the brain (see Higher olfactory centers). At the base of the sensilla, olfactory neurons are surrounded by support cells (also called accessory or auxiliary cells); these cells secrete the fluid filling the sensillum (lymph) and produce odorant binding proteins (OBPs). A direct role for OBPs in insect olfaction remains debateable (Larter et al. 2016; Xiao et al. 2019). OBPs are among the most abundant transcripts found in antennal tissues (Pitts et al. 2011b), and given their abundance, it is hypothesized they play a role in odor reception, most likely by binding to an odor molecule and transporting it to an odorant receptor (OR) (Venthur and Zhou 2018). In vitro studies of *An. gambiae* OBPs have identified OBPs that can bind a variety of odorants (Venthur and Zhou 2018). The most well characterized is AgOBP1, which based on in vitro assays, can bind the human odorant sulcatone, the repellent *N*, *N*-diethyl-meta-toluamide (DEET), and the animal odor indole (Biessmann et al. 2010; Murphy et al. 2013). RNAi knockdown of OBP1 leads to reduced electrical activity of whole antennae toward indole (Biessmann et al. 2010). However, it was not determined if OBP1 is expressed in sensilla that respond to these odorants, nor the effects of an *OBP1* mutant on olfactory or behavioral responses. The role of OBPs in olfactory responses has been most rigorously examined in *Drosophila melanogaster*. The *Drosophila* OBP LUSH enhances reception to the pheromone cis-vaccenyl acetate, in support of a role for OBPs in the transport of certain odor molecules (Gomez-Diaz et al. 2013). However, a comprehensive expression and mutant examination of the most abundantly expressed OBPs in the *Drosophila* antennae indicated that these OBPs played little role, if any, in odor reception (Larter et al. 2016; Xiao et al. 2019). In these experiments, up to 4 *obp* gene mutants were combined together to eliminate OBP expression in a sensilla, followed by single sensillar electrophysiological recordings of the olfactory neuron responses to a wide panel of odorants. These rigorous experiments demonstrated that olfactory neurons in sensilla lacking OBPs functioned normally, with some exceptions in which olfactory neurons exhibited increased odor responses. These data suggest that OBPs may not play a major role in odor reception, and may actually function as "sponges" to reduce odor concentrations in the lymph (Larter et al. 2016; Xiao et al. 2019). Further research is needed to determine the relative contribution of OBPs to odor perception in *Anopheles* mosquitoes (Biessmann et al. 2010; Murphy et al. 2013; Venthur and Zhou 2018).

Odorant molecules enter the lymph through multiple pores on the sensilla, allowing them to reach the olfactory receptors enriched on olfactory neuron dendrites. Transport through the lymph to the olfactory receptor might be aided by binding to OBPs, or the odorant might travel from the pore via pore tubules (structures found in many insect sensilla that extend from the wall pore to the dendritic membrane) (Steinbrecht 1997; Larter et al. 2016). Here, we describe olfactory receptor function and topology, followed by the anatomy of the peripheral olfactory appendages of adult *Anopheles* mosquitoes. Larval olfaction is also addressed in this section (see Larval olfaction).

Olfactory receptors and coreceptors

In mosquitoes, each olfactory neuron primarily expresses 1 of 3 classes of olfactory receptors: odorant receptors (ORs), ionotropic receptors (IRs), or gustatory receptors (GRs) (Figure 2). These receptors function as transmembrane ligand-gated ion channels comprised of various combinations of subunits. The OR and IR receptor groups contain a highly conserved coreceptor(s) paired with a subunit(s) tuned to respond to specific odorant ligands. ORs require an obligate *o*lfactory *r*eceptor *co*receptor called Orco; IRs utilize at least 3 different coreceptors (Ir8a, Ir25a, and Ir76b). There are no known olfactory coreceptors for GRs in mosquitoes, suggesting that

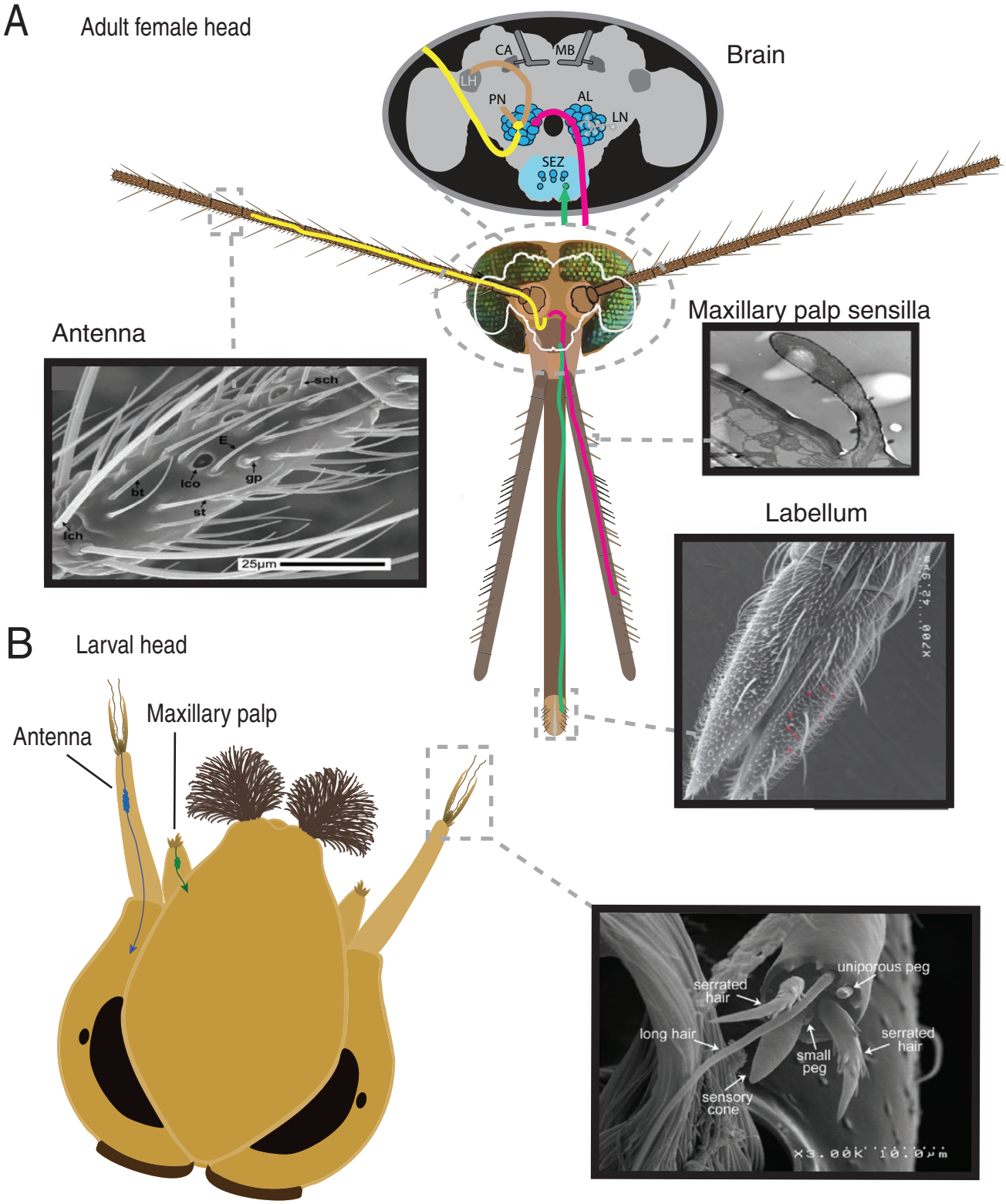

Figure 1. Organization of olfactory system in *Anopheles* mosquitoes. **(A)** The peripheral olfactory system in mosquitoes is composed of 3 sensory appendages/organs: a pair of antennae, a pair of maxillary palps, and one labella at the tip of the proboscis. Each of the sensory appendages is covered in specialized types of sensilla, which allow for detection of odors and lead to neuronal activation (see Figure 2 and text for more details). Olfactory neurons that originate in one of the sensory appendages (indicated by yellow, pink, and green lines in the mosquito head cartoon) innervate one of the 2 brain regions: the antennal lobes (AL) (from the antennae and the maxillary palps) and the subesophageal zone (SEZ) (from the labella). Olfactory local interneurons (LNs) shape the olfactory signal in the glomeruli of the antennal lobe that is transmitted to the olfactory projection neurons (PNs), which in turn project to higher olfactory centers: LH (lateral horn), CA (calyx) of the MB (mushroom bodies). The MB calyx is a dense dendritic region that collects olfactory inputs into an association (learning/memory) brain center and allows the insect to associate (remember) odors that coincide with pleasant or aversive stimuli. The LH is involved in influencing

either olfactory GRs do not require coreceptors, many different GRs function as coreceptors, or that multiple tuning receptors complex together. Here, we provide a brief overview of olfactory receptors and focus on the structural facets elucidated directly from *Anopheles* species. In-depth description of the primary olfactory receptor families across mosquito species has been reviewed elsewhere (Sparks et al. 2018). Recent studies suggest the coexpression of coreceptors in olfactory neurons in *Aedes* and *Drosophila* (Task et al. 2020; Younger et al. 2020); if this pattern also extends to *Anopheles* olfactory neurons remains to be determined.

Of the 3 olfactory receptor families, ORs have been the most studied. Each OR channel/complex is thought to be a heterotetramer consisting of subunits of Orco and odorant tuning receptor, ORx (Pask et al. 2011). These subunits are 7-transmembrane (7-TM) proteins with the N- and C-termini positioned intracellularly and extracellularly, respectively. Orco coreceptor is a highly conserved type of OR subunit that is found across insect species, whereas the ORx subunits tend to be highly divergent both within and between species (Rinker, Zhou, et al. 2013). *Anopheles gambiae* Orco was originally identified as OR7 through functional studies and homology to the *D. melanogaster* Orco (originally named Or83b), with 78% sequence similarity (Hill et al. 2002; Vosshall and Hansson 2011). Insect ORs represent the most diverse group of ligand-gated channels identified so far in nature. Both overall structure and individual amino acids play an important role in OR function in anopheline mosquitoes. Comparison of OR sequences of *Anopheles* and *D. melanogaster* revealed 3 distinct motifs (A, B, and C) in the C-terminus region in 76 of 79 (96%) known anopheline OR proteins (Miller and Tu 2008). These regions are hypothesized to function as protein–protein interaction sites used for Orco/ORx channel pore formation.

The second class of olfactory receptors, IRs, are composed of various subunits of odor binding IRs (IRx) and one of 3 IR coreceptors (IRco) (Sparks et al. 2018). IRx/IRco channel composition has yet to be fully characterized in anopheline mosquitoes. Current predicted structures include complexes of 3 IRco subunits with one IRx subunit (a similar heterotetramer structure seen in ORs) in 1:1 ratios (Pitts et al. 2017; Sparks et al. 2018; Li et al. 2019). Based on the sequence alignment of *An. sinensis*, the predicted topology of IRs includes 4 transmembrane domains (M1, pore loop, M2, and M3), along with an additional extracellular ligand-binding domain (LBD) (Benton et al. 2009; Li et al. 2019).

Although ORs only have one coreceptor (Orco), there are currently 3 identified IRco subunits: IR8a, IR25a, and IR76b. The structure of IR coreceptors differs compared with IRxs. Specifically, IRcos have an additional amino-terminal domain that directly follows the LBD, similar in structure to ionotropic glutamate receptors (iGluRs) found in other animals. Based on sequence homology, IRs are hypothesized to have evolved from iGluRs (Abuin et al. 2011; Wang et al. 2018; Li et al. 2019). Unlike iGluRs, due to the loss of glutamate-binding residues, IRcos are no longer able to bind glutamate. IRs are more highly conserved across insect species compared with ORs and GRs (Pitts et al. 2017; Wang et al. 2018).

Compared with ORs and IRs, little is known about the structure of GRs in *Anopheles* mosquitoes. GRs are hypothesized to be the ancestral receptor type that ORs evolved from as arthropods began their transition from aquatic to terrestrial environments (Eyun et al. 2017; Yan et al. 2020). Structurally, GRs retain the 7-TM topology found in ORs. Based on sequence alignment, despite the close relationship between GRs and ORs, the 3 conserved C-terminal motifs found in ORs are absent in GRs (Miller and Tu 2008). There is currently no published data on resolved protein architectures, limiting our knowledge on insect GR structures.

Despite advances in understanding the structure–function relationship in anopheline olfactory receptors, specific 3D structures of the olfactory receptors are unresolved. While obtaining the structures of transmembrane proteins is difficult, recent breakthroughs with cryogenic electron microscopy (cryo-EM) are bridging that gap. For example, cryo-EM allowed for the structure of the fig wasp *Apocrypta bakeri* Orco to be solved in a homotetramer formation (Butterwick et al. 2018). The structure of an ORx/Orco OR complex remains to be determined.

Various additional receptor and signaling molecules play important roles in insect olfaction. For example, several classes of transmembrane receptors may be involved in olfactory signal transduction in anopheline mosquitoes. These include transient receptor potential (TRP) channels which might respond to repellents such as citronellal (Kwon et al. 2010) or catnip/nepetalactone (Melo et al. 2021), epithelial sodium channels (DEG/ENaC)/pickpocket channels (ppk) found to amplify olfactory responses to pheromones in *Drosophila* (Ng et al. 2019), and sensory neuron membrane proteins found to mediate pheromone responses in *Drosophila* and Lepidoptera (Benton et al. 2007; Cassau and Krieger 2021). Odorant clearance or removal in the lymph by OBPs or odorant degrading enzymes will also affect olfactory signaling (Leal 2013). Similarly, numerous intracellular molecules probably function to regulate olfactory signaling, such as arrestins (Merrill et al. 2005) and other G-protein signaling components like $G\alpha_s$ (Deng et al. 2011). The role of these signaling molecules in *Anopheles* olfaction is an important yet under-explored area of research.

Sensory appendages: antennae

Both male and female adult *Anopheles* antennae have 13 segments called flagellomeres; at the base of each antenna is the donut-like pedicel, containing the Johnston's organ (the mosquito "ear") (McIver 1982). *Anopheles* antennae are sexually dimorphic. Males use audition to identify a mate, and thus most of their antennal sensory structures appear to be devoted to hearing (they have many more auditory hairs or fibrillae, and larger pedicels). Male olfactory sensilla are located only on the 2 distal segments of the antenna (Riabinina et al. 2016). In contrast, females have fewer auditory hairs and many more olfactory sensilla, which are distributed

innate olfactory behaviors. The function of the neurons in the *Anopheles* lateral horn have yet to be investigated, but they probably help to organize olfactory information into biological meanings (such as foraging odors or oviposition odors) (Dolan et al. 2019; Jeanne et al. 2018; Jefferis et al. 2007). Scanning electron microscopy (SEM) images reprinted with permission: antenna (Pitts and Zwiebel 2006), maxillary palp (Lu et al. 2007), and labella (Saveer et al. 2018). Sensilla shown in antennal SEM image: bt, blunt trichoid, E, similar to type E trichoid, gp, grooved peg, lch, large cheatica, lco, large coeloconic, sch, small cheatica, st, sharp trichoid. (**B**) *Anopheles* larvae use antennae and maxillary palps for olfaction. The sensory cone of the larval antenna is innervated by the dendrites of olfactory neurons. This sensory organ has a finely ridged porous surface with vacuoles at the basal region. These vacuoles are probably used for detection of soluble chemicals. When the antenna is stimulated with odorants, the molecules may diffuse through the cuticular covering, and then pass through the vacuoles to the dendrites (Zacharuk et al. 1971). Toward the outer part of the antenna tip is a uniporous peg organ—a basiconic-like appendage presumed to be gustatory. The brain targets for larval sensory organs are currently unknown. The SEM image of the larval antennal tip is from (Xia et al. 2008), Copyright (2008) National Academy of Sciences.

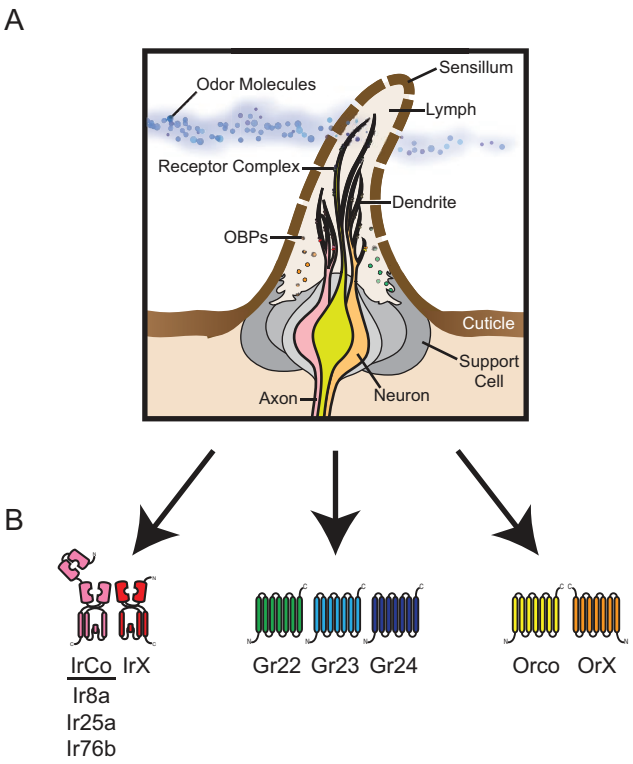

Figure 2. Anatomy of a sensillum and olfactory receptors in *Anopheles* mosquitoes. (**A**) Mosquito olfactory organs are covered in sensory hairs called sensilla. A cross-section of a typical sensillum is illustrated here. In *Anopheles* mosquitoes, each sensillum contains between 2 and 5 olfactory sensory neurons or OSNs (3 neurons are shown in this example). The dendrites of these neurons are found within the sensillum, whereas the axons send olfactory information to the mosquito brain. The neurons are surrounded by support cells (gray); these cells secrete the fluid or lymph filling the sensillum and produce OBPs. Odor molecules enter the sensillum through pores in the sensillar cuticle. OBPs may help in the transport of some odorants to the dendrites or act to sequester odor molecules. The dendrites express various olfactory receptor complexes. These complexes are ligand-gated ion channels that open when specific odor molecules bind to them, leading to the activation of the neurons. (**B**) Olfactory receptor complexes are composed of subunits from 3 broad classes or gene families: IRs, GRs, and ORs. Within each class, multiple subunits come together to form a functional ion channel. The IR complexes (left) consist of an IrX tuning receptor which confers ligand specificity on the neuron, in addition to one or more obligate coreceptors (Ir8a, Ir25a, and/or Ir76b). GR complexes (middle) consist of 3 subunits (Gr22, Gr23, and Gr24) which together sense carbon dioxide. OR complexes (right) consist of an OrX tuning receptor and the obligate coreceptor Orco.

throughout all but the most proximal flagellomere (flagellomere 1; McIver 1982), suggesting that olfaction is central to female physiology and behavior. There are 5 sensillar types on *Anopheles* antennae: sensilla chaetica, sensilla ampullacea, coeloconic sensilla, trichoid sensilla, and grooved pegs (McIver 1982). Three of these types are likely to be olfactory (Table 1). Both males and females appear to have all the same types of olfactory sensilla, but in different numbers and locations (Riabinina et al. 2016). For a comprehensive review of sensillar distributions in males and females, and detailed descriptions of sensillar types and subtypes in several *Anopheles* species, see (McIver 1982; Pitts and Zwiebel 2006; Qiu, van Loon, et al. 2006).

All *Anopheles* olfactory sensilla contain between 2 (in trichoid) and 5 (in large coeloconic) olfactory neurons (McIver 1982). The olfactory tuning receptor identity for most of these neurons is currently unknown. Olfactory neurons expressing the Orco coreceptor are found in trichoid sensilla (Pitts et al. 2004; Riabinina et al. 2016). Based on sensillar counts and genetic labeling of Orco+ cells, either most trichoid sensilla contain one Orco+ and one Orco– cell or else approximately half of trichoid sensilla contain 2 Orco+ cells each and the other trichoid sensilla express other receptors such as IRs (McIver 1982; Riabinina et al. 2016). It is currently difficult to distinguish these possibilities by histological means; new genetic reagents that label subsets of olfactory neurons might aid in this endeavor (as has been the case in *D. melanogaster*) (Couto et al. 2005; Fishilevich and Vosshall 2005; Kurtovic et al. 2007).

Little is known about IR expression in the antennae. Although the coreceptor Ir76b does not appear to overlap with Orco expression, the type(s) of sensilla housing Ir76b neurons were not identified (Pitts et al. 2017). Interestingly, Ir76b expression was later found to be closely associated with the expression of the ammonium transporter AgAmt, located in supporting cells surrounding Ir76b+ neurons. In *An. coluzzii* antennae, AgAmt was found in neurons and supporting cells in grooved peg and coeloconic sensilla, as well as in the palps and labella (Ye et al. 2020). It did not colocalize with Orco. This complex expression pattern prompts the question of AgAmt's role in these neurons and supporting cells. Ye et al. (2020) found ammonia-sensitive neurons not only in coeloconic sensilla, but also, surprisingly, in the capitate peg sensilla in the palps. Recent mutation of *AgAmt* revealed another surprise: the antennae of these mutant mosquitoes retain the ability to respond to ammonia odors (Ye et al. 2021). These data suggest AgAmt is unlikely to directly mediate ammonia detection in mosquitoes and suggest the molecular mechanism responsible for ammonia detection remains to be identified.

Given that both male and female *Anopheles* appear to have the same sensillar types, and express many of the same olfactory receptors, this suggests that both sexes are capable of sensing or responding to the same odors. However, since females probably have more sensilla and more neurons of each type, they may have a lower threshold of detection (be more sensitive to low concentrations of odorants) (e.g., Lu et al. 2007; Schymura et al. 2010). Transcriptomics studies also raise intriguing open questions such as the function of olfactory receptors expressed only in one sex or the chemosensory function of GRs expressed in the antennae (Pitts et al. 2011a).

In general, there are 2 broad challenges to connecting olfactory neuron identity to function in the *Anopheles* antenna: the first is the lack of a stereotypical organization of neuron types as found in other insects such as *Drosophila*, and the second is the sheer number of sensilla, neurons, and olfactory receptor genes (McIver 1982; Hill et al. 2002; Riabinina et al. 2016). Although many olfactory receptors (especially in the OR family) have been functionally characterized in heterologous expression systems (Carey et al. 2010; Wang et al. 2010; Pitts et al. 2017), few have been connected to antennal location or even sensillar type (Schymura et al. 2010; Schultze et al. 2013, 2014; Karner et al. 2015). Conversely, many electrophysiological experiments have been done on mosquito antennae with little to no knowledge of the underlying molecular mechanisms/identities of the neurons being recorded (van den Broek and den Otter 1999, 2000; Meijerink et al. 2001; Qiu, van Loon, et al. 2006). Although currently existing techniques such as calcium imaging in the periphery (Afify et al. 2019) in combination with heterologous expression and histology can begin to address some of these issues, new genetic tools to label and manipulate specific cell types are sorely needed.

Table 1. Olfactory sensilla in sensory organs of adult *Anopheles* mosquitoes

Sensory organ	Sensillar type	Sensillar class/subtype	Hypothesized function	Notes	References
Antenna	Sensilla chaetica		Mechanosensory		
	Sensilla ampullacea		Thermosensory	– Hypothesized to be small type of coeloconic-like sensilla – Not well studied	McIver (1982)
	Coeloconic sensilla	Small coeloconics	Thermosensory	– Common to all mosquito genera – Tunning receptor responding to cooling in the 3 coeloconic sensilla at the tip of the antenna identified	Greppi et al. (2020); McIver (1982)
		Large coeloconics	Olfactory	– Specific to *Anopheline* mosquitoes – Least abundant and studied of the antennal sensilla – Probably express IRs	
	Trichoid sensilla	Up to 5 morphological types/classes suggested in certain *Anopheles* species	Olfactory	– Most abundant olfactory sensilla – Unknown whether morphological class correlates with receptor expression and olfactory responses – Some morphologically similar sensilla can be distinguished physiologically after the female has taken a blood meal – Many express ORs; some may express IRs	Boo (1980); McIver (1982); Qiu et al. (2006b)
	Grooved pegs	Subtype A Subtype B	Olfactory and/or humidity sensing	– Not well studied – May express IRs	McIver (1982)
Maxillary palps	Sensilla chaetica		Mechanosensory		
	Campaniform sensilla		Mechanosensory		
	Capitate pegs		Olfactory	– Each peg has 3 neurons (A, B, and C) – A neuron responds to CO_2 and expresses GRs – B and C neurons express ORs	McIver (1982)
Labella	T1		Gustatory	– Sparse, largest sensilla on the labella	
	T2		Olfactory	– About 30 short sensilla with 2 OSNs/sensillum: large amplitude A neuron and small amplitude B neuron	Kwon et al. (2006)[a]; Riabinina et al. (2016); Saveer et al. (2018)

[a]Note reversed nomenclature of A and B neurons in Kwon et al. (2006).

Sensory appendages: maxillary palps

Of the *Anopheles* olfactory appendages, the maxillary palps are the simplest and most well characterized. Both male and female palps consist of 5 segments; in the males, the 2 most distal segments are expanded into a club (McIver 1982). As in the antennae, both male and female palps have the same types of sensory structures (Table 1), but their distribution and numbers are sexually dimorphic (McIver 1982). The only chemosensory sensillar type on the palps is the capitate peg sensillum (cp). Each is innervated by 3 olfactory neurons designated cpA, cpB, and cpC based on their characteristic spontaneous spike amplitudes (largest in A to smallest in C) (Lu et al. 2007; McIver 1982). The cpA neuron expresses Grs and responds to carbon dioxide (Lu et al. 2007), whereas the cpB and cpC neurons express Orco and a tuning OR (AgOr8 and AgOr28, respectively) and respond to 1-ocent-3-ol and a variety of odorants (Pitts et al. 2004; Lu et al. 2007; Pitts et al. 2011a; Riabinina et al. 2016). The cpA neuron expresses a complex of 3 GRs (*Gr22*, *Gr23*, and *Gr24*) necessary for CO_2 reception (Lu et al. 2007). Interestingly, recent work indicates that only Gr23 and Gr24 probably form the functional CO_2 receptor, with Gr22 acting to modulate the Gr23/Gr24 response toward CO_2 (Liu et al. 2020). In addition to CO_2, the cpA neuron is also excited and inhibited by many odorants (Lu et al. 2007; Tauxe et al. 2013; Coutinho-Abreu et al. 2019), and some of these odor responses are mediated by Gr22 (Liu et al. 2020), and possibly by coexpression of IRs (Younger et al. 2020).

Despite thorough anatomical, molecular, and physiological characterization, some questions about the *Anopheles* maxillary palps remain. For example, transcriptional profiling (quantifying RNA expression using techniques such as qRT-PCR or RNAseq) of the palps in males and females revealed additional potential olfactory receptors, including nine IRs (3 found in both sexes, plus 2 female-specific IRs and 4 male-specific ones), and 2 additional GRs (one specific to each sex) (Pitts et al. 2011a). Since the only known chemosensory sensilla on the palps are the capitate pegs, this suggests that there may be some neurons that coexpress IRs with ORs or GRs; this idea is supported by recent findings in the palps of *Aedes* mosquitoes (Younger et al. 2020). Furthermore, a small proportion of Orco+ neurons in the palps apparently do not express either *AgOr8* or *AgOr28*, suggesting that they may instead express different tuning receptors (Lu et al. 2007).

Finally, if the primary role of the palps is the integration of host cues, such as CO_2 and octenol, as has been suggested (Lu et al. 2007), then what is the role of the palps in *Anopheles* male physiology/behavior? Interestingly, animals are not the only source of CO_2 in the mosquito's environment: plants also emit CO_2 (Peach et al. 2019). One possibility is that males (and females) use CO_2 in combination with other plant volatiles to choose a nectar source (see Role of olfaction in foraging). Whether males can detect these plant volatiles with their maxillary palps, and then integrate this information with CO_2, has not, to our knowledge, been investigated.

Sensory appendages: labella

The mosquito proboscis consists of a fascicle or bundle of 6 stylets with a retractable sheath (the labium); the stylets include 2 maxillae, 2 mandibles, a labrum, and a hypopharynx (McIver 1982; Wahid et al. 2003). In females, the maxillae saw through the skin of the host, whereas the mandibles separate the tissue; in both males and females, the hypopharynx secretes saliva, and the labrum is the food canal for both nectar and blood (Wahid et al 2003; Choo et al. 2015). Of the 6 stylets, only the labrum has sensilla, which probably house gustatory neurons and possibly sensory neurons for other modalities (e.g., mechanosensory, thermosensory, and/or hygrosensory) (McIver 1982; Maekawa et al. 2011; Jové et al. 2020). The stylets are enclosed by a retractable and flexible labium, at the tip of which is the labellum, composed of 2 lobes (McIver 1982; Choo et al. 2015). When a female mosquito takes a blood meal, the stylets pierce the skin of the host; the labium bends out of the way, with the labellar lobes remaining on the surface of the skin (Choo et al. 2015). Interestingly, even though only female mosquitoes bite, male proboscises are morphologically quite similar to those of the females. *Anopheles* males have all 6 stylets, but their mandibles and maxillae are shorter than females' and less adapted for piercing (Wahid et al. 2003). Of the various proboscis structures, the labellum is the only one to have been implicated in olfaction.

Like the other peripheral appendages, *An. gambiae* labella are covered in sensilla, including a small population of T2 olfactory sensilla (Table 1). Each T2 sensillum contains 2 olfactory neurons (Kwon et al. 2006; Riabinina et al. 2016; Saveer et al. 2018). There are around 60 olfactory neurons per labellar lobe, approximately 45 of which express Orco (Pitts et al. 2004; Riabinina et al. 2016). These numbers suggest that there may be approximately 15 T2 neurons, which express other olfactory receptors, such as IRs (see Olfactory receptors and coreceptors). As the identities of the ligand-binding receptors have not been mapped to the vast majority of the labellar olfactory neurons, this is a fruitful avenue of future research. To date, only one olfactory receptor, AgOr6, has been confirmed in labellar olfactory neurons (Kwon et al. 2006). RNAseq experiments have revealed that a number of ORs, IRs, GRs, and OBPs are expressed in the labella (Saveer et al. 2018). These experiments demonstrated that some OR transcripts enriched in the labella are lowly expressed or even absent in the other olfactory appendages, suggesting an interesting potential target for specific, close-range modulation of *Anopheles* olfactory behavior to prevent biting.

Although currently there is no receptor-to-neuron map of the OSNs in the labella, efforts have been made to characterize their functional profiles (Kwon et al. 2006; Saveer et al. 2018). Using the gustatory T1 sensilla as landmarks, the labellum was subdivided into 4 anatomical zones (numbered 1 through 4 from distal to proximal) (Saveer et al. 2018). Interestingly, OSNs in different zones appear to have different odorant response properties: for example, the A neurons in zone 3 are more narrowly tuned than the A neurons in the other zones (Saveer et al. 2018).

One present challenge is dissociating the relative behavioral contributions of labellar OSNs from olfactory neurons in the antennae and maxillary palps. For example, many of the strongest activators of labellar neurons, such as indole and acetophenone (Saveer et al. 2018), are known to also activate OSNs in the antennae (Qiu, van Loon, et al. 2006) and palps (Lu et al. 2007), respectively. Experiments in which the different olfactory appendages are ablated or inactivated, as has been done in the *Culex* proboscis (Choo et al. 2015), might help elucidate the behavioral significance of the labellar olfactory neurons.

Higher olfactory centers: general organization

OSNs in peripheral tissues serve as neuronal odorant detectors and function to directly convey olfactory signals into the brain. The insect brain organizes these neuronal signals into perceptions which guide behaviors. Recent work identified 2 *An. coluzzii* brain regions innervated by olfactory neurons: the antennal lobes (ALs) and the subesophageal zone (SEZ) (Riabinina et al. 2016).

A central dogma of insect olfactory neurons is that antennal and maxillary palp olfactory neurons that express the same type of tuning olfactory receptor converge their axons into a shared region of the brain called a glomerulus. The collection of all glomeruli makes up the AL. For example, there are ~10 olfactory receptor neurons that express the AgOR2 receptor across the *Anopheles* antennae, with roughly 0–2 AgOR2-expressing neurons in each flagellomere (Schymura et al. 2010). Although it remains to be experimentally verified, the axons for each of these neurons are expected to target the same glomerulus. This converging of olfactory neurons expressing the same olfactory receptor toward a common brain region serves to amplify the olfactory signals from the periphery as they enter the brain.

An olfactory glomerulus is formed by the olfactory circuits that innervate the glomerular region: 1) the axons of the OSNs which serve as the input to the circuit, 2) the dendrites of the olfactory projection neurons, which serve as the output for the circuit, and 3) the processes of the local neurons, which innervate only the AL glomeruli to help shape the olfactory signal that is transmitted from olfactory neuron to projection neuron. From a study examining dye-filled projection neurons in *Anopheles* mosquitoes (Ignell et al. 2005), it appears these projection neurons share features found in other insects such as *Drosophila* (Jefferis et al. 2007) and moths (Homberg et al. 1988) and primarily target 2 regions of the brain: the calyx of the mushroom bodies and the lateral horn (Figure 1).

Higher olfactory centers: ALs

The *An. coluzzii* AL is comprised of 67–70 glomeruli as determined by staining for a synaptic protein (bruchpilot, detected by the *Drosophila* nc82 antibody) (Riabinina et al. 2016), although variability between brain samples regarding the size and shape of glomeruli was noted (Riabinina et al. 2016; also see Anton and Rospars 2004). Variability of ALs might be due to technical issues of dissection, brain staining, and imaging, or they might reflect biological variability between individuals. The male AL contains a similar number of identifiable glomeruli as the female (67–68) but is smaller in size due to the proportionally fewer olfactory neurons of each class innervating and forming each glomerulus (Riabinina et al. 2016). The female AL measures ~170 000 μm^3, which is roughly 1.9 times larger

than the male AL at ~90 000 µm^3 (Riabinina et al. 2016). Genetic labeling of Orco+ olfactory neurons revealed that approximately half of the AL glomeruli are innervated by Orco+ olfactory neurons (33 in males and females) (Riabinina et al. 2016). This finding suggests that the remaining glomeruli are probably innervated by IR neurons (IRNs) or by CO_2-sensing GR neurons (GRNs). Note, the original investigation of the *An. gambiae* AL structure mis-classified a group of glomeruli as a Johnston's organ center (Ghaninia et al. 2007); improved neurogenetic and staining methods in *An. coluzzii* identified this region as containing many glomeruli, as well as being innervated by Orco+ olfactory neurons (Riabinina et al. 2016).

Anopheles olfactory neurons that originate from the antenna project only to the ipsilateral AL (the AL on the same side as the antenna) (Riabinina et al. 2016). This contrasts *D. melanogaster*, in which an antennal olfactory neuron generally first targets the ipsilateral AL, and also sends a projection to the matching glomerulus on the contralateral AL (the AL on the other side of the brain) (Dobritsa et al. 2003). *Anopheles* olfactory neurons that originate from the maxillary palp, however, do target both ALs: the 3 olfactory neurons from the capitate peg sensilla target the ipsilateral AL, and also send contralateral projections to the matching glomeruli on the contralateral AL (Anton et al. 2003; Ghaninia et al. 2007; Riabinina et al. 2016).

The 2 Orco+ olfactory neurons in the capitate peg sensilla of the maxillary palps (Or8- and Or28-expressing neurons) target 2 posterior glomeruli in the *Anopheles* AL (Ghaninia et al. 2007; Riabinina et al. 2016). Dye filling experiments of the maxillary palp suggest that the third olfactory neuron in capitate peg sensilla, which expresses the Gr22/Gr23/Gr24 complex and is sensitive to CO_2, may target up to 3 posterior glomeruli (Riabinina et al. 2016). It remains to be determined if subsets of the olfactory GRNs target one of the 3 glomeruli (and hence are organized into 3 different grouping of GRNs), or if the same olfactory neuron can target all 3 glomeruli. Recent evidence suggests that Gr22 is not required for detection of CO_2, but instead enhances the ability of Gr23/Gr24 to respond to CO_2 (Liu et al. 2020). This observation suggests that changes in expression of Gr22 might lead to changes in the ability of the neuron to respond to CO_2 (and some other odorants), which might be further reflected by potentially differential targeting of AL glomeruli.

Higher olfactory centers: SEZ

The ~45 Orco+ olfactory neurons from the *An. coluzzii* labella do not target the AL, but instead target the SEZ of the brain (Riabinina et al. 2016). One report using dye labeling of the labella suggested some labellar olfactory neurons innervate the AL (Kwon et al. 2006), but further genetic labeling of Orco+ neurons and dye labeling could not verify these results (Riabinina et al. 2016). The SEZ region of an insect brain is often associated with gustatory behaviors (Wang et al. 2004; Miyazaki and Ito 2010). Genetic labeling of Orco+ olfactory neurons in *An. coluzzii* revealed the presence of 8 glomerular structures in the SEZ in both male and female brains (Riabinina et al. 2016). It remains to be determined if labellar olfactory neurons in other insects also target the SEZ region of the brain. The implications of olfactory innervation in a gustatory region suggest that olfactory and gustatory sensory information could be directly integrated by neurons within this brain area. Integration of olfactory and gustatory sensory modalities in the human brain gives rise to the perception of flavor, and an intriguing hypothesis is that *Anopheles* mosquitoes may be using gustatory and olfactory neurons on the labella to similarly examine the "flavor" of a host-landing site and use this to inform on its decision to bite. Similarly, it is possible that favorable host odors detected by the labella might be interpreted by the SEZ brain region as appetitive tastes.

Larval olfaction

In contrast to adult *Anopheles*, not much is known about olfaction in larvae and pupae. There is no current evidence for chemosensory-driven behavior in *Anopheles* pupae. Pupae do not forage and do not seem to have functional antennae or palps (Montell and Zwiebel 2016). Fully functioning adult sensory appendages need to develop inside their heads, whereas the larval olfactory appendages are removed by the last molt. Pupae appear to predominantly rely on visual cues to interact with their environment.

Anopheles larvae navigate their chemical world using olfactory receptors expressed in the antennae and maxillary palps (Pitts et al. 2004). Similar to other dipteran insects, the antennae of larval *An. gambiae* mosquitoes are bilaterally symmetrical, projecting anteromedially from the lateral surface of the head. Morphologically, the larval antenna is composed of chemosensory structures including a sensory cone and a peg organ (Figure 1B). These structures play a crucial role in olfaction and presumably gustation, respectively (Zacharuk et al. 1971).

The sensory cone is proposed to be the main olfactory organ in the larvae of *An. gambiae* and is innervated by ~12 bipolar neurons expressing Orco (Xia et al. 2008). The larval chemosensory structures of *An. coluzzii* are very similar between males and females (Riabinina et al. 2016). Notably, the median number of Orco+ cells identified in the male and female larval antennae is twice that of the maxillary palps (Riabinina et al. 2016). This pattern of expression suggests that in *Anopheles*, antennae might be more important than maxillary palps in larval odorant detection.

Larval antennae express 12 putative AgOrs and the obligate coreceptor, Orco (Carey et al. 2010; Wang et al. 2010; Xia et al. 2008). Further investigations of the 12 reported AgOrs identified 4 (AgOr37, AgOr40, AgOr52, and AgOr58) that are speculated to be larval specific (Xia et al. 2008), although some have been detected at low levels in adult tissues (Maguire et al. 2020). Recently, upregulation of AgOr52 in the male and female adult *An. gambiae* during mating was reported, suggesting that this OR might not be larval specific (Mozūraitis et al. 2020). Heterologous expression of the 12 candidate AgOrs using either *Xenopus* oocytes or *Drosophila* empty neuron system revealed some corresponding ligands that activate these receptors (notably indole and benzaldehyde), which activate AgOr2 and AgOr10 in the larvae and adult female *An. gambiae* (Xia et al. 2008; Carey et al. 2010; Wang et al. 2010). Nonetheless, the ligands for AgOr52 and AgOr58 have not yet been identified. Apart from the AgOrs, AgIr76b has been shown to be crucial for behavioral responses of *Anopheles* larvae to butylamine (Liu et al. 2010). The role of AgIrs in larval olfaction has been largely understudied.

Contrary to adult *Anopheles* mosquitoes that are free-living on land, the larvae inhabit confined aquatic environments such as puddles, pools of standing water, and drainage ditches (Muema et al. 2017). Given the nature of the larval habitat, to survive, larvae must find sufficient food resources while avoiding numerous dangers, including predators (e.g., *Toxorhynchites* larvae, dragonfly nymphs, and some aquatic Hemiptera) (Ohba et al. 2010), and toxic compounds.

Anopheles larvae have evolved chemosensory detectors of harmful odorants and display strong aversive behavioral responses to compounds such as acetophenone produced by *Pseudomonas* bacteria (a compound associated with mammalian disease; Xia et al.

2008), DEET (a potent adult mosquito repellent) (Xia et al. 2008), and a mosquito larval repellent VUAA1 (2-((4-ethyl-5-(pyridine-3-yl)-4H-1,2,4-triazol-3-yl)thio)-N-(4-ethylphenyl)acetamide) (Yang et al. 2020). A compound from *Bacillus thuringiensis israelensis* bacteria has also been shown to be highly toxic to mosquito larvae (Benelli et al. 2016). Additionally, compounds such as sulcatone and dimethyl disulfide (possibly produced by the bacteria or the larvae themselves) are present in water with high larval densities. It is unclear if /how some of those potentially toxic compounds are detected by the *Anopheles* larvae to cause aversive behaviors.

Olfactory responses may also allow larvae to navigate to and locate food resources. The larvae of *An. gambiae* can detect products derived from organic decay (of both plant and animal origin) including indole, 2-methylphenol, and 4-methylcyclohexanol (Xia et al. 2008). Although products of organic decay might indicate the presence of a food source to the mosquito larvae, there is also evidence that larvae have a symbiotic relationship with indole-producing bacteria such as *Serratia* and *Pantoea* (Villegas and Pimenta 2014). Little is known about how the detection of this bacteria-derived volatile influences larval behavior.

Electrophysiological recordings of the larval sensory cone have recently revealed the population response properties of larval olfactory neurons (Sun, Liu, Baker, et al. 2020). Larval olfactory neurons responded to a wide array of odorants, primarily thiazoles, alcohols, and heterocyclics, and different subsets of neurons could be identified that respond strongly to many of the key odorants described above. Future studies will probably link chemoreceptors to their distinct larval olfactory neurons and investigate how odor coding functions in this simpler system.

Role of olfaction in *Anopheles* adults

Role of olfaction in foraging

Both male and female mosquitoes rely on sugar-feeding from plant and other nectar sources (Foster 1995; Müller et al. 2010) for nutrition (Baker and Baker 1973; Foster 1995; Rivera-Pérez et al. 2017; Peach and Gries 2020) and energy, driving processes such as metabolism, flight, and mating (Foster 1995; Manda et al. 2007; Nyasembe and Torto 2014). During these sugar-foraging states, mosquitoes exhibit plant- and nectar-seeking behaviors guided by olfactory and gustatory cues to selectively locate and feed on carbohydrate sources of specific host plants (Impoinvil et al. 2004; Manda et al. 2007; Gouagna et al. 2010; Müller et al. 2010).

Plants and other sugar sources release volatile organic compounds (VOCs) including terpenes, benzenes, esters, aldehydes, and alcohols (Knudsen et al. 1993; Nyasembe and Torto 2014). Some of these VOCs influence olfaction-guided mosquito foraging, though their effects are often species specific and dependent on blend composition (Nyasembe et al. 2012; Nyasembe and Torto 2014). There is significant overlap in plant preference between both sexes, but differential responses and some distinct plant preferences exist (Healy and Jepson 1988; Manda et al. 2007; Müller et al. 2010; Nikbakhtzadeh et al. 2014). For example, *An. gambiae* s.s. females are more likely to fly upwind in the presence of certain plant odors and enter the plant-baited traps more frequently than males (Nikbakhtzadeh et al. 2014). Furthermore, some flowering plants are uniquely attractive to females (Müller et al. 2010). A complete characterization of sex-specific mosquito responses and preferences for plants and other carbohydrate sources is lacking.

Olfactory discrimination of suitable plants and nectar sources is species specific. *Anopheles gambiae*, *Aedes aegypti*, and *Ae. ochraceus* mosquitoes detect unique volatile compound classes from their preferred host plants (e.g., sesquiterpenes and alkenes), as well as general plant attractants (e.g., monoterpenes β-myrcene and (*E*)-β-ocimene) (Nyasembe et al. 2018). Although a complete profile of plant semiochemicals involved in olfaction-mediated nectar-seeking is lacking, several volatile compounds from plant headspaces that elicit responses in *Anopheles* species have been identified (Nyasembe and Torto 2014). For example, *An. arabiensis* responds to extracted volatiles from the flowering plant *Achillea millefolium*, with a cyclic or bicyclic monoterpene tentatively identified as the active compound (Healy and Jepson 1988). In *An. gambiae* s.s. females, 6 electroantennographic detection (EAD) active plant compounds (hexanal, limonene, (*Z*)- and (*E*)-linalool oxide, β-pinene, (*Z*)- and (*E*)-β-ocimene, and (*E*)-β-farnesene) are involved in olfaction-mediated attraction to several plant species (Nyasembe et al. 2012). Of those compounds, linalool oxide (detected by AgOr50 and AgOr20) (Carey et al. 2010; Wang et al. 2010) was more efficient in capturing female *An. gambiae* s.s. than the full 6 component blend under field conditions (Nyasembe et al. 2015; Jacob et al. 2018). Combining linalool oxide with plant monoterpenoids β-pinene, β-ocimene, and L-limonene can also increase the efficiency of trapping *An. pharoensis*, but not *An. arabiensis* or *An. funestus* (Jacob et al. 2018). Thus, some plant preferences are species specific and are probably influenced by ecological niche, season, and food source availability.

Another consideration in mosquito olfaction is how yeast and microbiota inhabiting plants may influence plant VOC emission and thus the cues received and interpreted by mosquitoes (Madden et al. 2018; Barredo and DeGennaro 2020; Klaps et al. 2020). Yeast-generated CO_2 (≥200 ml CO_2/min) is sufficient to attract female mosquitoes, including *An. gambiae* s.s. and *An. arabiensis* (Smallegange et al. 2010; Aldridge et al. 2016). Traps with CO_2 also enhance mosquito attraction compared with plant inflorescences alone (Nyasembe, Tchouassi, et al. 2014; Nyasembe et al. 2015; Aldridge et al. 2016; Peach et al. 2019), suggesting that CO_2 is also a potential cue for mosquitoes during nectar-seeking.

Although many VOCs eliciting behavioral and electrophysiological responses in mosquitoes have been identified, the context in which odors are interpreted is equally important. This context-dependent olfactory-mediated behavior depends on several factors, including volatile released ratios, blend composition, and the physiological state of the mosquito. Different plant species often vary in odor compound composition as well as released ratios of VOCs, and mosquitoes use both these qualitative and quantitative differences in host odor profiles to discriminate during plant-foraging (Nyasembe et al. 2012, 2018). Modifying blend ratios of the active components of plants alters the attractiveness to female *An. gambiae* s.s. mosquitoes, in some cases making the synthetic blend even more attractive than the natural plant host (Nyasembe et al. 2012). There is also evidence that attractive components can become repellent at different ratios (see Repellents and attractants of host-seeking mosquitoes) (Jacob et al. 2018), and many plant species attractive to *An. gambiae* also contain known repellents (Nyasembe and Torto 2014; Lutz et al. 2017).

There is also significant overlap in odor profiles between human volatiles attractive to mosquitoes and headspace volatiles found in plants foraged upon by mosquitoes. For example, 1-octen-3-ol, nonanal, butanoic acid, 2-methylpropionic acid, 2-methylbutanoic acid, 3-methylbutanoic acid, benzoic acid, hexanoic acid, (−)-α-pinene, benzaldehyde, and acetophenone are all odorants

found either in the odor bouquet of inflorescences or in human skin or breath (Peach et al. 2019). The mechanisms of host discrimination remain unclear, but context of chemical cues combined with other sensory modalities probably allow mosquitoes to differentiate between plant and blood host.

Role of olfaction in mating

Anopheline mosquitoes are among various insect species that mate in swarms. During swarm formation, males aggregate at dusk using visual markers (Marchand 1983). Females then enter the swarm, pair with a mate, and leave in copula for in-flight mating to occur (Charlwood and Jones 1979). Olfaction appears to have a limited role in this process, which instead predominately involves visual and auditory cues.

Although males primarily rely on visual cues known as swarm markers to select sites for swarm formation, it is still unclear how female mosquitoes locate these swarms. In some species, there is evidence that male-produced aggregation pheromones may attract females (Cabrera and Jaffe 2007; Fawaz et al. 2014) and a recent study showed that *An. arabiensis* and *An. gambiae* male mosquitoes produce and release aggregation pheromones that attract both male and female mosquitoes to the swarm and enhance mating success (Mozūraitis et al. 2020). The identified 5-component blend of 3-hydroxy-2-butanone (acetoin), 6-methyl-5-hepten-2-one (sulcatone), octanal, nonanal, and decanal was released by *An. gambiae* and *An. arabiensis* males in significantly higher amounts during swarming. These compounds also elicited increased mating in 3 other Anopheline species (*An. coluzzii*, *An. merus*, and *An. funestus*) (Mozūraitis et al. 2020). All 5 of these compounds are found in human odor profiles, highlighting the emerging question of how olfaction-guided mosquito responses may differ in various contexts.

Role of olfaction in host-searching and blood-feeding

Once a female mosquito has obtained enough food resources, either before or after mating (Charlwood et al. 2003), she begins searching for a suitable host to blood-feed (Figure 3). This blood meal is crucial for egg development. Females orient themselves toward potential hosts by detecting and responding to various long-range (e.g., CO_2 gas and volatile components of host body odor) and short-range (e.g., body heat, relative humidity, visual contrast) cues. The final decision to bite is made after landing and assessing host suitability using contact cues (e.g., skin moisture and surface tastants). Although different sensory modalities are involved in mosquito–host attraction (Bowen 1991; Raji and DeGennaro 2017), olfaction is arguably the most important, as it initiates the entire sequence of events and mediates mosquito–host interactions (Bowen 1991; Takken 1991; Takken and Knols 1999).

Role of olfaction in mosquito attraction to human scent

Since mosquitoes predominantly rely on olfaction while host-searching, variation in human scent affects mosquito attraction, making some humans more likely to be approached and bitten than others (Knols et al. 1995; Qiu, Smallegange, et al. 2006). Host-searching females exploit 3 sources of chemical cues of human scent: exhaled breath, body odor, and urine (Takken 1991). Human odor is a complex blend of hundreds of volatiles (including organic fatty acids, ketones, aldehydes, esters, and alcohols), which varies from person to person (Krotoszynski et al. 1977; Bernier et al. 2000; Curran, Rabin, Furton 2005; Curran, Rabin, Prada, et al. 2005; Qiu, Smallegange, et al. 2006; Dormont et al. 2013). This interindividual scent variation is influenced by genetics, diet and environmental factors, and use of personal/cosmetic products (Curran, Rabin, Furton 2005). Some elements of attraction to blood-feeding insects might be heritable in humans as shown in non-malarial vectors. Evidence from the volatiles emanating from identical twins highly correlates with their attractiveness to *Ae. aegypti* mosquitoes (Logan, Cook, et al. 2010; Fernández-Grandon et al. 2015).

Attractiveness to mosquitoes also changes with age, body size, and physiological state of the host. *Anopheles gambiae* attraction increases with host age probably because of increased skin surface area, resulting in more emission of volatile compounds (Busula, Verhulst, et al. 2017). As expected, more bites are recorded on young children and adults than on infants (Bryan and Smalley 1978; Carnevale et al. 1978). Furthermore, certain components of body odor (nonanal, dimethylsulphone, and benzothiazole) might also be specifically associated with aging (Gallagher et al. 2008). Moreover, pregnant women are more attractive to *An. gambiae* and *An. arabiensis* mosquitoes compared with nonpregnant ones during sleep. Heightened attractiveness during pregnancy might be linked to increased body temperature and increased release of skin volatiles (Lindsay et al. 2000; Ansell et al. 2002; Himeidan et al. 2004). Thus, human attractiveness to mosquitoes is dependent on our prevailing physiological states, in addition to body type/size.

Certain dietary items consumed by humans may guide females to locate and select those people as hosts. *Aedes albopictus* and *An. gambiae* females exhibit a strong affinity (activation, orientation, and landing) to people within 15 min of them drinking alcohol (Shirai et al. 2004; Lefevre et al. 2010). Similarly, increased attractiveness to *An. stephensi* and *An. gambiae* mosquitoes is observed within 3 h of those eating bananas (Paskewitz et al. 2018). Conversely, consumption of garlic or vitamin B supplements provides no protection against mosquito bites, as shown in *Ae. aegypti* and *An. stephensi* (Ives and Paskewitz 2005; Rajan et al. 2005). The effect of diet might be confounded with host physiological state (CO_2 release, body temperature, and metabolic changes) and vector's physiological responses (detection and encoding by the olfactory system).

Modification of human scent with personal care products (e.g., soap, perfume) can temporarily repel or attract mosquitoes. Some of these products incorporate synthetic and natural repellents (see Repellents and attractants of host-seeking mosquitoes) that ward off host-searching mosquitoes. For example, certain underarm deodorant compounds (e.g., isopropyl tetradecanoate) can repel *An. coluzzii* (Verhulst et al. 2016), whereas methyl dihydrojasmonate and lilial components of perfume fragrances effectively repel *Culex quinquefasciatus* mosquitoes (Zeng et al. 2018). These products can suppress or activate mosquito attraction via 2 modes of action. First, they can directly affect the mosquito sense of smell. Second, they can affect the skin microflora dynamics, which then modify human scent to repel or attract mosquitoes (Verhulst et al. 2016).

One of the most important components of exhaled breath for mosquito attraction is CO_2 gas, along with water vapor and VOCs. This long-range cue acts as a behavioral activator for mosquitoes and it synergizes responses to other host cues (Reeves 1951; Healy and Copland 1995; Mboera and Takken 1997; Takken and Knols 1999; Hinze et al. 2021). Since CO_2 levels in human exhaled breath are about 100 times more than the atmospheric levels (Gillies 1980), female mosquitoes are behaviorally activated by detecting small

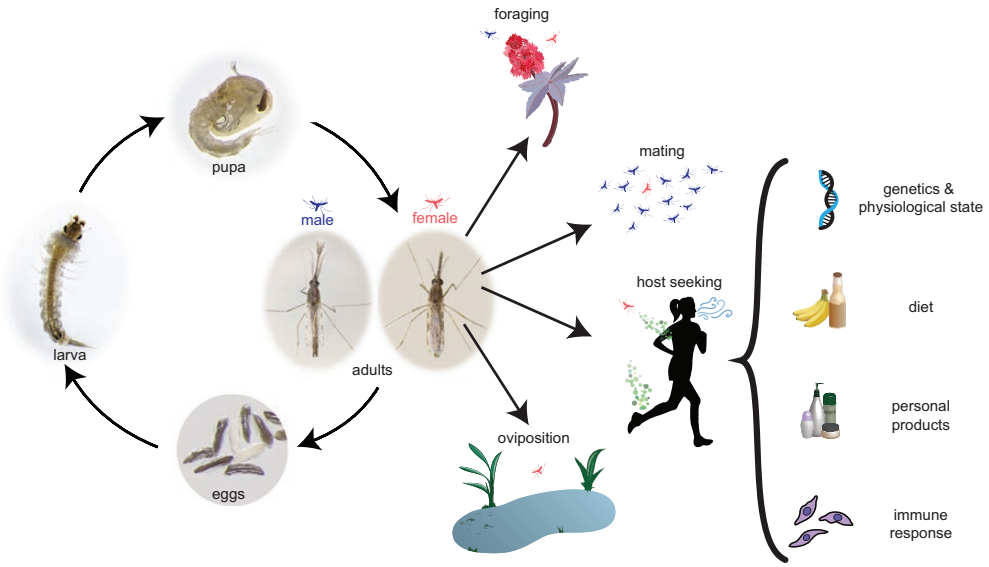

Figure 3. Life cycle of *Anopheles* mosquitoes and olfactory-guided behaviors of adults. **Left**: *Anopheles* mosquitoes go through 4 main developmental stages. Eggs (bottom) are laid individually in water and remain on the surface due to egg floats. After approximately 24 h, they hatch into larvae (left), which will feed on bacteria and detritus in the water as they grow and molt through 4 instars. This process takes about 1 week, depending on environmental conditions. With the final molt, larvae become pupae (top), which no longer feed but are still motile. Adults (right) emerge from pupae approximately 24 h later. Male mosquitoes are indicated in blue, female mosquitoes in light red. **Right**: Both male and female mosquitoes must forage for nectar from plants (top). Mosquitoes mate in swarms of males (second from top), into which females fly. Only females engage in host-seeking and blood feeding behaviors (second from bottom), as well as oviposition or egg-laying (bottom). Bracket: many factors affect the attractiveness of a human host to a female *Anopheles* mosquito, including genetic influences on skin flora and emitted odor volatiles, physiological state such as age and pregnancy, diet, the use of personal hygiene products and mosquito repellents, and immune response, including infection with the *Plasmodium* parasite (bottom).

changes in CO_2 concentration (Webster et al. 2015). Detection of CO_2 gas motivates females to find additional specific cues indicative of host presence. Responses to these other host-derived cues, which may or may not be attractive by themselves, can be enhanced by the addition of CO_2. This synergy is exemplified by increased attractiveness to known components of human odor (lactic acid, 1-octen-3-ol, nonanal, and carboxylic acids) when mosquitoes are presented with CO_2 (Dekker et al. 2005; Njiru et al. 2006; Qiu, van Loon, et al. 2006; Smallegange and Takken 2010; van Loon et al. 2015).

Mutant *Ae. aegypti* females that lack a functional *orco* olfactory coreceptor (and thus do not respond to many human odors) regain the attractiveness (but not the preference) to humans when CO_2 is added (DeGennaro et al. 2013). Similar findings were reported in *An. coluzzii* mosquitoes using Orco-defective neurons or *orco* mutants (Maguire et al. 2020; Sun, Liu, Ye, et al. 2020). These findings suggest that CO_2 is needed to trigger a host-seeking state in mosquitoes and to integrate multiple cues involved in host-searching (McMeniman et al. 2014; Potter 2014).

Once in closer vicinity to a host, CO_2 becomes less important to mosquitoes in identifying host's suitability. At this stage, body odor predominantly activates the olfactory receptors, located on all 3 sensory organs of the mosquito's peripheral olfactory system (Montell and Zwiebel 2016) (see Sensory appendages). Carboxylic acids contained in sweat and VOCs above the skin are the main component of human odor. Alcohols, aldehydes, aromatic hydrocarbons, amines, esters, ketones, sulfides, and thiols are also present (Bernier et al. 2000; Dormont et al. 2013). These compounds, responsible for attraction in *An. gambiae*, are either naturally produced by gland secretions (i.e., sweat) or are a byproduct of bacterial growth on the skin surface (Braks et al. 2000). Of these sweat and skin odorants, several important *Anopheles* attractants include 1-octen-3-ol (octenol) (Lu et al. 2007), lactic acid (Braks and Takken 1999), ammonia (Smallegange et al. 2005; Qiu, van Loon, et al. 2006; Ye et al. 2020), nonanal, and carboxylic acids (e.g., hexenoic and octenoic acids) (Knols et al. 1997; Meijerink and van Loon 1999; Costantini et al. 2001). Interestingly, these studies found that these odorants, when tested alone, are minimally (if at all) attractive, yet become attractive in the presence of CO_2; this reflects the ability of CO_2 to activate olfactory host-seeking behaviors, and the synergy between CO_2 and host-odors in olfactory attraction. Generally, acetates, alcohols, and ketones are detected by ORs, whereas acids and amines are detected by IRs.

Of the hundreds of known components of human scent, it is clear that only a specific fraction is crucial for host-seeking mosquitoes. Although some of those components elicit physiological and behavioral responses when presented on their own, it is the blend and concentration of those individual components that modulate the responses to host cues in mosquitoes, including *An. gambiae* (Dekker et al. 2002; Smallegange et al. 2005; Qiu et al. 2011; Majeed et al. 2016). This is supported by recent neurogenetic work in *Ae. aegypti* mosquitoes that examined the composition of volatile odorants from humans or animals (rat, dog, guinea pig, quail, sheep), and also the mosquito's olfactory responses to these complex blends (Zhao et al. 2020). Odor profiles were overlapping among humans and nonhuman animals, with human odors enriched for sulcatone, geranylacetone, decanal, undecanal, and acetoin and showing lower relative abundances of hexanal and heptanal (Zhao et al. 2020). Interestingly, a single population of olfactory neurons were more strongly activated by human odor blends than animal odor blends, with decanal and undecanal being the odorants most strongly activating "human" olfactory neurons; conversely, a different olfactory neuron population was more strongly activated by

non-human animal odors (Zhao et al. 2020). These data suggest that the brain of the *Aedes* mosquito can gauge the relative "humanness" of a complex odor blend and possibly use this to guide host-seeking behaviors. It will be critical to determine whether the *Anopheles* olfactory system shares this coding strategy.

Role of olfaction in the control and plasticity of host-searching and preference

The degree of plasticity in host preference must weigh the trade-off between obtaining a blood meal and avoiding defensive host behaviors as well as other selective pressures in the environment (host diversity, density, and distribution). Although it is widely accepted that *An. gambiae* mosquitoes show an extreme form of specialization for human hosts, they will readily bite other mammals if humans are unavailable (Lefevre et al. 2009).

It is likely that the organization of the mosquito nervous system underlies host preference. To date, a few studies have investigated the genetic basis of host preference by comparing the genetic makeup of closely related mosquito species with different host preferences. Although *An. gambiae* specifically prefer human hosts, *An. quadriannulatus* strongly prefer nonhuman hosts. This preference may be in part determined by the olfactory system. Comparison of the antennal transcriptome of the 2 species revealed differential enrichment of chemosensory genes in the *Obp*, *Ir*, and *Or* gene families (Rinker, Zhou, et al. 2013).

Interestingly, all *Obp* transcripts were more abundant in the antennae of *An. gambiae*, with the total reads per kilobase million (RPKM) of detectable *Obps* twice that of *Obps* found in *An. quadriannulatus*. In contrast to the *Obps*, the *Irs* and the *Ors* exhibited widespread variation in transcript abundances. Specifically, *Irs* varied significantly between the 2 species, with 27 of the 30 showing detectable differences in abundance. Although these species share the same suite of genes encoding ORs, they also showed major deviations in *Or* transcripts. Although there were no *Ors* whose antennal expression was specific to *An. gambiae*, 84% of tuning *Ors* showed significant differences in expression, with 16 *Ors* enriched more than 2-fold in one of the species (Rinker, Zhou, et al. 2013). These differences in chemosensory gene abundance in different *Anopheles* species might mediate host preference, for example by increasing the proportion or sensitivity of OSNs in the periphery that respond to human odors (in anthropophilic strains) or animal odors (in zoophilic strains).

Although the genetically predetermined preference for humans is established before pupal emergence, responsiveness to host odors depends on the mosquito's age. Females generally refrain from host-seeking and biting ~1-day postemergence (Takken et al. 1998; Omondi et al. 2019). Once maturation is complete, females show a gradual increase in responsiveness to a host at 2–6 days old (Takken et al. 1998). This gradual change in the valence of human odor (from slightly aversive to attractive) as the female mosquito ages from days 1 to 4, is correlated with changes in *Or* expression. These selected *Ors* (e.g., *Or1*, *Or2*, *Or75*, which increase ~1.5- to 3-fold) respond to human odorants. During this period, the sensitivity of odorant receptor neurons tuned to human odorants also slightly increases (Omondi et al. 2019). These changes in olfactory sensitivity demonstrate an age-dependent response to host odors. The expression levels of *Ae. aegypti* chemoreceptors were also found to vary during different life stages (Hill et al. 2021).

Once fully mature and ready to blood-feed, mosquitoes host-seek according to an endogenous timing system. This "circadian clock" system synchronizes many physiological and behavioral processes to occur at a specific time of the day. Blood-feeding behavior in *An. gambiae* is under circadian regulation as shown under laboratory conditions using membrane feeders (Das and Dimopoulos 2008) and live hosts (Rund et al. 2013). Under natural conditions, *An. gambiae* mosquitoes are primarily nocturnal biters, exhibiting the highest frequency of biting behavior between 9 pm and midnight (Mathenge et al. 2001). There is a clear time-of-day modulation of olfactory sensitivities that is responsible for this pattern of *Anopheles* host-searching and biting activity. Specifically, olfactory responses to host odors are stronger a few hours after than before darkness (Rund et al. 2013). It remains to be determined if these changes in olfactory physiology gate the timing of biting behavior.

Blood-feeding as a critical step in disease transmission

In hematophagous mosquitoes, egg production is cyclic and is initiated by a blood meal, which contains necessary proteins for normal egg development and maturation. As shown in *Ae. aegypti*, major components of human blood such as gamma-globulins, albumin, and hemoglobin (Leeman et al. 2018) are crucial to allow hormonal activation of egg development and increased fecundity (Kogan 1990; Zhou et al. 2007). The same blood proteins are probably important for other vectors including *Anopheles* mosquitoes.

Following a blood meal, female mosquitoes show a reduction in host-seeking and biting behavior. The initial inhibition probably results from abdominal distension produced by blood ingestion, as demonstrated in *Ae. aegypti* mosquitoes (Klowden and Lea 1979). This initial short-term reduction in biting is followed by a suppression of host-seeking and biting for several days (Duvall et al. 2019). *Aedes aegypti* will generally not bite again until their next gonotrophic cycle (time between blood meal and oviposition). Similarly, *An. gambiae* mosquitoes experience this initial host-seeking and biting reduction 12 h after a blood meal (Klowden and Briegel 1994). Although their initial feeding suppression is probably similar to that of *Ae. aegypti*, the olfactory system may also cause a short-term suppression of host-seeking. Transcripts for individual tuning *AgIrs* and *AgOrs* in the antennae show a general pattern of depletion up to 12–48 h following a blood meal, which may reduce antennal odorant receptivity in vivo (Fox et al. 2001; Rinker, Pitts, et al. 2013).

After the initial suppression of host-seeking behavior, unlike *Ae. aegypti* females (Klowden 1981), *An. gambiae* host-seek and bite multiple times during a single gonotrophic cycle. The total caloric reserves, carried over from the larval stages, are considerably lower in teneral (newly emerged) Anophelines than in Culicines (Briegel 1990b). This difference in energy resource may explain how *Ae. aegypti* mosquitoes can complete a gonotrophic cycle with a single blood meal, whereas *An. gambiae* cannot. Moreover, this effect seems to be body size dependent. Small *An. gambiae* individuals need more blood meals than large ones (Lyimo and Takken 1993; Takken et al. 1998) (Briegel and Hörler 1993). Larger mosquitoes contain relatively more metabolic reserves at emergence, and they can produce and lay more eggs per gonotrophic cycle than smaller individuals (Briegel 1990a). Oviposition in *Anopheles* (see Role of olfaction in oviposition) takes place within 48–60 h after a blood meal (Briegel and Hörler 1993), which marks the end of the gonotrophic cycle. At this point, the female mosquito probably regains her olfactory responsivity to host odors and is ready to host-search again (Klowden 1981).

The need to obtain blood to complete egg development makes blood-feeding a critical step in disease transmission. Although blood-feeding itself probably relies more on gustation and

mechanosensation than on olfaction (see Sensory appendages), it marks a transitional phase between plant, host, and oviposition site seeking states of an adult female. Each of these stages is characterized by olfactory changes that maximize the chances of mosquito success. The female mosquito's reliance on olfaction is critical for her success, but this dependence can be exploited by other organisms. Specifically, the finely tuned olfactory system of host-seeking female *Anopheles* is vulnerable to parasite-induced changes in host attractiveness and behavioral manipulation by the malaria-causing *Plasmodium* (see Plasmodium host and Vector manipulation sections). Fortunately, mosquito olfactory sensitivity can also be exploited by humans in vector management and prevention of disease transmission (see Olfaction and vector management).

Role of olfaction in oviposition

After blood-feeding, female mosquitoes develop eggs and subsequently search for an oviposition site (Figure 3). Gravid (egg carrying) females use olfactory cues, among others (visual and tactile), to assess an oviposition substrate and ensure its suitability for their offspring (e.g., presence of food, mosquito immature stages, predators, and pathogens) (Bentley and Day 1989; Afify and Galizia 2015; Day 2016). Mosquito larvae feed on plant infusions and microorganisms that live on them (Merritt et al. 1992), and accordingly, cues of plant infusions and microorganisms have been shown to attract egg-laying mosquitoes. For example, females of *An. arabiensis* and *An. coluzzii* showed an oviposition preference to volatile extracts from certain grasses such as the antelope grass (*Echinochloa pyramidalis*) and hippo grass (*Echinochloa stagnina*) (Asmare et al. 2017). In addition, females of *An. gambiae* prefer ovipositing on substrates from their natural breeding sites that contain live microorganisms over sterilized substrates (Sumba et al. 2004). The presence and density of immature mosquito stages can be interpreted by the gravid female differently: limited numbers can indicate a suitable oviposition substrate, whereas higher densities of these stages can indicate competition (Afify and Galizia 2015; Day 2016). A good example for this is the oviposition preference of *An. gambiae* females for water that contains low densities of *C. quinquefasciatus* eggs. As the density of *C. quinquefasciatus* eggs increases in the water, this water becomes repellent to *An. gambiae* gravid females (Wachira et al. 2010). The developmental stage itself also seems to have an effect on oviposition preference; *An. coluzzii* gravid females prefer to lay eggs on water containing conspecific first-instar larvae but avoid laying eggs on water with fourth-instar larvae, possibly to limit chances of intraspecific competition or cannibalism (Mwingira et al. 2020). Gravid mosquitoes in these studies were not allowed to touch the test water (Wachira et al. 2010; Mwingira et al. 2020), suggesting that attraction/repellency toward mosquito immature stages is olfaction dependent. Gravid mosquitoes are also known to avoid oviposition sites that contains their predators. For example, *An. gambiae* s.s. females avoid laying eggs on water that contains the predatory fish *Gambusia affinis* and *Carassius auratus* (Chobu et al. 2015).

Mosquitoes use their sensory appendages to detect oviposition cues. Volatile compounds of different plant odors attract *An. arabiensis* gravid females and activate their antennae in electroantennogram experiments (Wondwosen et al. 2016, 2017, 2018). Oviposition repellent volatiles from overcrowded and resource-deprived *An. coluzzii* larvae (dimethyl disulfide, dimethyl trisulfide, and 6-methyl-5-hepten-2-one) also elicit an electrophysiological response in the antennae, maxillary palps, and labella of gravid *An. coluzzii* mosquitoes (Suh et al. 2016). Some *An. gambiae* ORs, such as AgOr10, strongly respond to oviposition attractants such as 3-methylindole (Carey et al. 2010). In support of an important role for OR signaling in oviposition, gravid *An. coluzzii* females mutant for *orco* demonstrated significant defects in attraction to olfactory cues associated with oviposition site selection (Sun, Liu, Ye, et al. 2020). Changes in sensitivity to oviposition and host cues are believed to be regulated across the mosquito gonotrophic cycle, to reflect changes in mosquito behavior. Electrophysiological responses in *An. gambiae* to oviposition odorants increase after blood-feeding and are accompanied by a decrease in response to host odorants (Qiu et al. 2013). This regulation of odorant sensitivity reflects changes in antennal chemosensory gene expression following blood-feeding (Rinker, Pitts, et al. 2013).

Anopheles olfaction and malaria transmission

The *Plasmodium* blood parasite, vectored by *Anopheles* mosquitoes, causes malaria that affects over 200 million people worldwide, with 400 000 deaths annually (WHO 2019). For successful transmission, this parasite depends on both its vertebrate host and mosquito vector to complete different phases of its lifecycle (Figure 4). Only *P. ovale*, *P. malariae*, *P. knowlesi*, *P. falciparum*, and *P. vivax* infect humans, with the latter 2 being the deadliest and most prevalent (CDC 2020).

There is regional variation in incidence and seasonality of malaria, with high prevalence in Sub-Saharan Africa and Asia. Climatic conditions, host susceptibility, as well as distribution and biological characteristic of vectors (e.g., dominant mosquito species, vectorial capacity) determine this variation (Kiszewski et al. 2004). In Africa, the *An. gambiae* complex (including *An. gambiae* s.s., *An. coluzzii*, and *An. arabiensis*) and *An. funestus* codominate, whereas *An. darlingi* dominates the Americas (Sinka et al. 2010, 2012; Autino et al. 2012; Kyalo et al. 2017; Irish et al. 2020). In the Asian-Pacific region, multiple mosquito species including *An. stephensi* and *An. culicifacies* complex are recorded as major malaria vectors (Sinka et al. 2012; Suwonkerd et al. 2013). Although there are ~480 formally recognized species of *Anopheles*, only 30–40 of them are capable of transmitting malaria under natural conditions and are thus considered dominant vector of malaria (Autino et al. 2012; Harbach 2013; CDC 2020).

Transmission of *Plasmodium* parasite is also dependent on the female mosquito's physiology and her olfactory-guided host-searching abilities. A female bites at least twice to transmit malaria: first ingesting of the gametocyte stage of *Plasmodium* from an infected host and then passing the sporozoite stage to a new uninfected host (Figure 4). The time between these bites is ~12–14 days to allow sporozoites development in the *Anopheles* mosquito midgut and migration to the salivary glands (Shaw and Catteruccia 2019). To enhance this transmission, *Plasmodium* may manipulate both the vector's propensity to bite and the host's body odor to increase the chances of interaction between the vector and the host.

Plasmodium host odor modification for increased mosquito attraction

To increase the likelihood of a female biting an infected host, the *Plasmodium* parasite modifies the host's odor to make the host more attractive to blood-seeking mosquitoes. This modification can include changes in the relative amounts of certain components of the host emanate (exhaled breath, specific body parts, and whole-body odors) (De Moraes et al. 2014; Berna et al. 2015; de Boer et al. 2017)

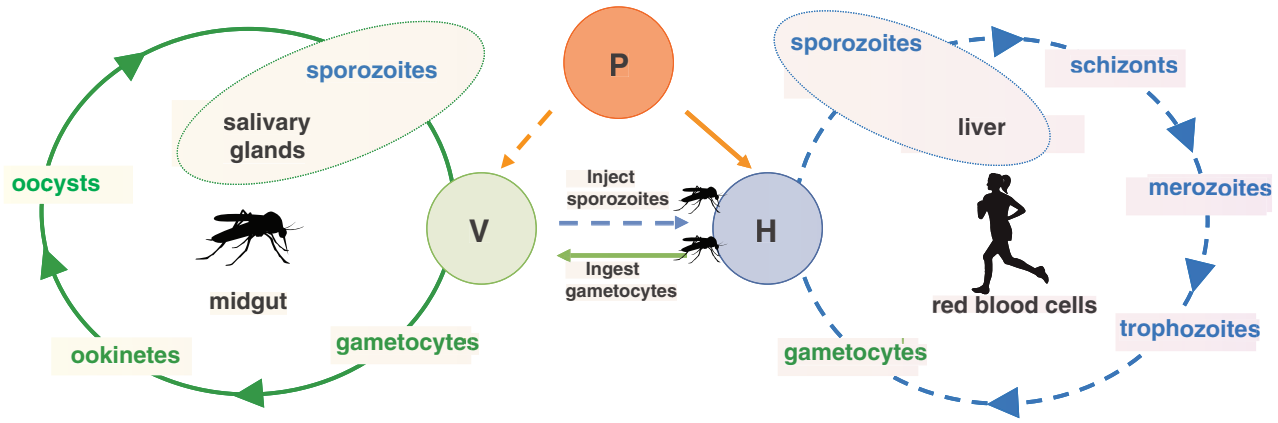

Figure 4. Malaria-causing *Plasmodium* needs both a female mosquito vector and a vertebrate host to complete its lifecycle. Different stages of *Plasmodium* parasite (orange) are infective to mosquito vectors (green) and human hosts (blue). A female mosquito needs to blood-feed at least twice to transmit malaria and she can only be infected following ingestion of the sexual gametocyte stage of *Plasmodium* when she bites an infected host (solid green lines and arrows). Once inside a mosquito, male and female *Plasmodium* gametocytes form motile zygotes (ookinetes), which invade the mosquito midgut wall. Ookinetes develop into oocysts, which eventually rupture releasing sporozoites, which invade the mosquito's salivary glands. Sporozoites are the only stage of *Plasmodium* that can infect human hosts after a female mosquito injects them along with her saliva during a blood meal (dashed blue lines and arrows). Sporozoites invade the liver and mature into schizont, which release merozoites. Merozoites in turn invade red blood cells, where *Plasmodium* goes through repeated cycles of asexual reproduction. Merozoites produce immature ring stages, which develop into mature trophozoites and produce schizonts again (which will go through the cycle and infect new red blood cells). Small proportion of the immature ring stage trophozoites develop into male and female gametocytes (*Plasmodium* sexual stages). The human host now harbors the only *Plasmodium* stage infective to female mosquitoes taking a blood meal. The critical step for infection of both mosquito vectors and vertebrate hosts is highly dependent on mosquito olfaction. P, *Plasmodium* parasite; V, mosquito vector; H, human host.

and the production of novel components (Kelly et al. 2015; Emami et al. 2017).

Notable increases in aldehydes (e.g., heptanal, octanal, nonanal) (Robinson et al. 2018) and thioethers (Berna et al. 2015) and decreases in some ketones (e.g., 6-methyl-5-hepten-2-one) (de Boer et al. 2017) occur in host body odor and breath during malaria infection. These changes result in different volatile profiles of symptomatic (overall decrease in emission) and asymptomatic (overall increase in emission) malaria carriers compared with uninfected individuals (De Moraes et al. 2018). These malaria-induced changes in the host odor profile do not depend on the infection itself, but rather on the developmental stage of the *Plasmodium* parasite inside the host. Specifically, marked changes in host odor and mosquito attraction are observed between infectious (sexual gametocytes) and noninfectious (asexual schizont) stages of malaria in vertebrate hosts. Since the gametocyte stage of *Plasmodium* needs to complete its lifecycle in a mosquito (Figure 4), the parasite may increase the attractiveness of hosts that carry this infection stage. In dual-choice laboratory and semifield studies, gametocyte-carrying individuals (both human and nonhuman) attract more *Anopheles* mosquitoes compared with those carrying asexual stages of *Plasmodium* or those being malaria-free (Lacroix et al. 2005; Batista et al. 2014; De Moraes et al. 2014; Busula, Bousema, et al. 2017; Robinson et al. 2018). Therefore, malaria induces stage-specific changes in the host volatile profile to increase attraction of mosquitoes to hosts most likely carrying the infectious stage of malaria, ensuring transmission.

Additionally, certain volatiles such as terpenes in host odor and exhaled breath profiles are induced by *Plasmodium* itself (Kelly et al. 2015; Emami et al. 2017) or result from an interaction between host and parasite metabolic pathways (Berna et al. 2015; Kelly et al. 2015; de Boer et al. 2017; Schaber et al. 2018). *Plasmodium*-produced isoprenoid metabolic precursor, (E)-4-hydroxy-3-methyl-but2-enyl pyrophosphate (HMBPP), is responsible for increased release of CO_2 gas, aldehydes, and monoterpenes (including α- and β-pinene) in cultured human red blood cells. Similarly, certain thioether breath components (e.g., (Z)-1-methylthio-1-propene and 1-methylthiopropane) increase from barely detectable to 50–100 times higher over the course of malaria infection, only to drastically decline just hours later upon antimalaria drug administration. Consequently, parasite modifications of the host emanate profiles can enhance mosquito attraction.

Moreover, changes in human odor profile during malaria infection are amplified by skin microflora, involved in the production of volatiles that mosquitoes respond to during host-searching (Braks et al. 1999; Busula, Verhulst, et al. 2017; de Boer et al. 2017). Malaria-associated compounds detected during early infection (e.g., 2-and 3-methylbutanal and 3-hydroxy-2-butanone) are produced by skin bacteria, and not by *Plasmodium* (de Boer et al. 2017). The release of these compounds by the skin bacteria may explain the long-lasting effects on host odor profile (and probably mosquito attraction) following immune challenge with malaria, even after gametocytes have been cleared from the blood (i.e., when the host is no longer infective) (de Boer et al. 2017).

Plasmodium vector manipulation for increased host-seeking and biting

Once a female *Anopheles* has blood fed on a *Plasmodium*-infected host, behavioral and physiological modifications may occur if she becomes infected by the parasite (Hurd 2003; Stanczyk et al. 2017). These modifications, dependent on the *Plasmodium* developmental stage the vector carries, are manifested as altered biting motivation and efficiency. Mosquitoes harboring the nontransmittable oocyte stage of *Plasmodium* reduced both the biting persistence and host attraction to humans (Anderson et al. 1999; Cator et al. 2013). Only when the transmittable sporozoite stage was present did increases in host attraction and the number of close interactions (landing, biting persistence, blood-feeding frequency, and meal size) occur in the females (Koella et al. 1998; Anderson et al. 1999; Cator et al. 2013; Smallegange et al. 2013). Consequently, chances of malaria transmission to new hosts would be higher by mosquitoes carrying

the infective stages of parasite due to increased host attraction compared with those carrying the noninfective stages. These behavioral modifications by *Plasmodium* appear to be species specific as some *Anopheles* show no changes in host-seeking behavior (Vantaux et al. 2015; Nguyen et al. 2017). Thus, the sensory bias of infected mosquitoes (i.e., vector manipulation by the *Plasmodium* parasite) might not be universal. Other nonhost-seeking behavioral modification of the *Plasmodium*-infected mosquitoes is observed during foraging and nectar feeding, which suggests broad changes in the mosquito olfactory system (Nyasembe, Teal Peter, et al. 2014).

Changes in olfaction, which ultimately result in these modified behaviors, originate from altered sensitivities and detection thresholds of the sensory appendages (Cator et al. 2013; Stanczyk et al. 2019), and changes in protein expression in the brain (Lefevre et al. 2007; Emami et al. 2017). The changes in olfactory sensitivities, and thus behavioral responses to host cues, are species specific and seem to depend on mosquito–*Plasmodium* pairings. This pattern is exemplified by differential antennal and maxillary palp responses of malaria infected and uninfected species of *Anopheles* to human volatile extracts including known mosquito attractants (e.g., lactic acid, 1-octen-3-ol, benzothiazole) (Cator et al. 2013; Grant et al. 2013; Stanczyk et al. 2019). Moreover, physiological responses to some of those odorants can decrease at the noninfective oocyte stage, but increase once the sporozoites invade the salivary glands of the females to make them infectious (Cator et al. 2013). Similarly, differential expression of several proteins involved in metabolism and neuronal function is observed in the head proteome of *An. gambiae* infected with the sporozoite (i.e., infective) stage of *P. berghei* (Lefevre et al. 2007). Upregulation of genes with modulatory functions in neural synapses can also occur after ingesting a blood meal containing a *Plasmodium*-produced compound (Emami et al. 2017). Together, these findings suggest that the presence of *Plasmodium* in female mosquitoes may lead to changes in gene expression, olfactory sensitivity, and behavior to favor malaria parasite transmission.

It is still unclear if the changes in mosquito behavior and olfactory sensitivities result from direct manipulation of the vector by *Plasmodium* or if they are associated with an immune response and overall physiological adjustment to infection. Similar behavioral effects and altered olfactory sensitivity experienced by a *Plasmodium*-infected mosquito can be induced by immune challenge by other microorganisms. Direct injection of heat-killed *Escherichia coli* bacteria in *An. stephensi,* after a blood meal from an uninfected host, mirrors the behavioral and neurophysiological modifications of infection with *Plasmodium* (Cator et al. 2013). Similarly, even if *An. gambiae* females are not infected after ingesting a *Plasmodium*-containing blood meal, they respond less to certain odorants, indicating modification of olfactory organ sensitivity following immune challenge (Stanczyk et al. 2019). These data suggest that changes in the mosquito olfactory system might not be exclusively induced by *Plasmodium*.

Olfaction and vector management

Integrated vector management (IVM) is the rational use of multiple resources and strategies for the purpose of vector control. This concept includes the use of different strategies of intervention, including chemical (e.g., indoor residual spraying) and biological methods (e.g., using natural enemies) instead of a single-intervention approach to successfully control insect vectors (Beier et al. 2008). In this review, we focus on strategies exploiting mosquito olfaction to reduce vector–human interaction to decrease chances of disease transmission. These strategies include the use of odorants to attract or repel host-seeking mosquitoes, disrupt mosquito mating, and prevent mosquito oviposition (Wooding et al. 2020).

Repellents and attractants of host-seeking mosquitoes

To successfully prevent mosquito biting, a push/pull strategy can be used. This strategy takes advantage of mosquito dependence on olfaction to find humans for blood-feeding, and uses repellents to "push" mosquitoes away from humans or their dwellings and "pull" them with attractants toward alternative hosts or toward traps to kill them (Cook et al. 2007).

Mosquito repellents are a group of odorants that prevent mosquitoes from locating their hosts (e.g., humans) and hence disrupt blood-feeding. Repellents can be applied directly on the skin or clothes (topical repellents) or used to protect an area or space from biting mosquitoes (spatial repellents) (Debboun et al. 2014). There are 2 types of topical repellents: 1) plant-based repellents such as lemongrass oil, eugenol (extracted from clove oil), nepetalactone (extracted from catnip), and para-methane 3-8, diol (PMD; extracted from lemon–eucalyptus essential oil), and 2) synthetic repellents such as DEET, Picaridin, and IR3535 (Debboun et al. 2014).

Plant-based repellents have been shown to work against *Anopheles* mosquitoes (Maia and Moore 2011; Debboun et al. 2014; Afify and Potter 2020). Nepetalactone (the active repellent ingredient in catnip), for example, appears to repel many insects, including *An. coluzzii* mosquitoes (Melo et al. 2021). Interestingly, nepetalactone may act by directly stimulating TRPA1 receptors: TRPA1 directly activates heterologously expressed *Ae. aegypti* and *Drosophila* TRPA1 receptors, and *Ae. aegypti* and *Drosophila* TRPA1 mutants are no longer repelled by nepetalactone (Melo et al. 2021). It remains to be determined if nepetalactone also activates *Anopheles* TRPA1 receptors to mediate repellency to catnip in *Anopheles* mosquitoes. Nonetheless, most natural repellents evaporate quickly after application and therefore offer relatively short protection time (Logan, Stanczyk, et al. 2010; Maia and Moore 2011).

On the other hand, synthetic repellents like DEET are less volatile (Spencer et al. 1979), making DEET the gold standard in long-lasting mosquito repellents (Debboun et al. 2014). There are different hypotheses on repellents' mode of action. A repellent could act directly on the mosquitoes by activating chemoreceptors on their sensory appendages to elicit a repellent behavior (Boeckh et al. 1996; Ditzen et al. 2008; Syed and Leal 2008; Afify et al. 2019; Afify and Potter 2020) or by modulating OR activity in response to attractive odorants (Bohbot and Dickens 2010; Pellegrino et al. 2011). Repellents could also act at a physical level by reducing the volatility of odorants on human skin and preventing them from reaching the mosquito (Syed and Leal 2008; Afify et al. 2019). Mosquito repellents might also work in more than one of these modes of action (Bohbot et al. 2011). The modes of action for mosquito repellents are species specific (Afify and Potter 2020). In *An. coluzzii*, plant-based repellents have been shown to strongly activate chemoreceptors on the antennae, probably leading to a repellent olfactory signal that drives repellent behaviors. In contrast, synthetic repellents (DEET, Picaridin, IR3535) probably do not activate *An. coluzzii* chemoreceptors as a mechanism for mediating repulsion as close-range olfactory behavioral assays with these repellents revealed no olfactory repulsion (Afify and Potter 2020). Odor-dependent activity monitoring of Orco-positive olfactory neurons instead suggests that these synthetic repellents may function primarily to mask the olfactory responses triggered by attractive odorants (Afify et al. 2019). The likely olfactory mechanism is by decreasing the volatility of these odorants (Syed and Leal 2008; Afify et al. 2019). The search for

safe, efficient, and long-lasting new repellents can benefit from recent advances in understanding how currently used repellents function.

Repellents can also be used to prevent mosquito biting in a 3D space (spatial or area repellents). These spatial repellents are either volatile enough to release in the air at ambient conditions or require active aerosolization or applying heat to volatilize the active ingredients (e.g., mosquito coils, candles, and torches) (Debboun et al. 2014). Spatial repellents can be plant based (like some topical repellents) (Maia and Moore 2011) or synthetic (like pyrethroids) (Debboun et al. 2014). Pyrethroids are frequently used insecticides with high knockdown activity that also repel mosquitoes at sublethal doses (Debboun et al. 2014). Laboratory studies showed that some pyrethroids repel *Anopheles* mosquitoes on contact (irritant effect), whereas others have a noncontact repellent effect, suggesting that they work through olfaction (Chareonviriyaphap et al. 1997; Dusfour et al. 2009; Kawada et al. 2014). In field studies, pyrethroid compounds have been shown to reduce house entrance and increase house exiting of *Anopheles* mosquitoes, suggesting that they can play a significant role in malaria vector control (Grieco et al. 2000; Kawada et al. 2008).

To lure mosquitoes toward traps, attractive odorants involved in host finding, such as CO_2 (Mboera et al. 2000; Rueda et al. 2001; Cooper et al. 2004; Njiru et al. 2006), ammonia (Njiru et al. 2006), and 1-octen-3-ol (Rueda et al. 2001; Cooper et al. 2004; Njiru et al. 2006), have been used to successfully capture *Anopheles* mosquitoes in field trials. Although these attractants can work individually, the combination of multiple attractants has been shown to increase their effectiveness in attracting *Anopheles* mosquitoes (Rueda et al. 2001; Cooper et al. 2004; Smallegange et al. 2005; Njiru et al. 2006). However, an odorant blend as attractive as a human host has not yet been identified.

The attraction of mosquitoes to various people can differ (see Role of olfaction in mosquito attraction to human scent), and an approach to identify new insect repellents has been to identify human odorants that reduce attraction and to utilize these chemicals as insect repellents (Logan et al. 2008; Logan, Stanczyk, et al. 2010). A number of human-derived odorants were identified to reduce *An. gambiae* mosquito attraction, including decanal, octanal, 6-methyl-hepten-2-one (also known as sulcatone), and geranylacetone, and mixtures of these odorants provided strong protection in arm-in-cage biting assays (Logan, Stanczyk, et al. 2010). Since these chemicals are typically found on human skin, they are more likely to be safe for human use and could provide promising alternatives to plant-derived repellents.

The response to attractive odorants has been shown to be concentration dependent in *Anopheles*. High concentrations of the attractants 1-octen-3-ol and benzaldehyde were found to be repellent to *An. coluzzii* (Afify and Potter 2020). In addition, higher concentrations of these odorants activate more antennal olfactory receptor neurons than lower concentrations, suggesting that high concentrations of normally attractive odorants may activate repellent-sensing neurons or globally activate many olfactory neurons leading to a repellent-like effect (Afify and Potter 2020).

Vector management: mating disruption

The knowledge about the olfactory aspect of mosquito mating is limited (see Role of olfaction in mating). Aggregation pheromones play a role in *Ae. aegypti* mating (Fawaz et al. 2014) and have also been recently identified in *Anopheles* (Mozūraitis et al. 2020). These aggregation pheromones could be used to attract mosquitoes into traps or disrupt mosquito mating (Vaníčková et al. 2017). Male *Anopheles* mosquitoes show some attraction to human odorants, suggesting that these volatiles may play a role in swarm formation and mating (Foster and Takken 2004). In addition, male *Anopheles* mosquitoes are more attracted to nectar volatiles than to human odorants (Foster and Takken 2004). These volatiles can be used to attract and capture male mosquitoes before swarming and thereby disrupt or decrease mating (Foster and Hancock 1994).

Vector management: oviposition site selection disruption

A push/pull approach can also be applied using mosquito oviposition cues. Oviposition repellents can prevent *Anopheles* mosquito egg laying around human dwellings, and oviposition attractants can be used to direct gravid mosquitoes into traps (Seenivasagan et al. 2019; Schoelitsz et al. 2020). Additionally, oviposition cues can be used for mosquito control in an autodissemination approach, where oviposition attractant is used to lure gravid mosquitoes into traps treated with the insect growth regulator pyriproxyfen. These gravid mosquitoes subsequently transfer the insect growth regulator to their natural oviposition sites, where the pyriproxyfen kills mosquito larvae. The benefit of this approach is that it enables broader coverage of pyriproxyfen and treats harder to reach mosquito habitats. This method was first developed to control container-breeding mosquitoes, such as *Ae. aegypti* (Itoh et al. 1994; Devine et al. 2009), and was later successfully tested in semifield studies against *Anopheles* mosquitoes (Lwetoijera et al. 2014, 2019; Mbare et al. 2014). The use of oviposition cues in push/pull and autodissemination approaches can complement other olfaction-based methods to efficiently limit *Anopheles* mosquito biting and malaria transmission.

Conclusion

To be a vector of malaria, the *Anopheles* mosquito must first locate, identify, and distinguish a human in its environment. If an *Anopheles* female mosquito bites a *Plasmodium*-infected person, it too can become infected, develop *Plasmodium* parasites, and spread this infection to other humans with her next bite. Before infection spreads, the female mosquito cycles through a number of behaviors such as foraging, mating, and ovipositing. As discussed in this review, each of these steps involves olfaction. We suggest that interfering with *Anopheles* olfaction is a potent strategy for modifying mosquito behavior, with each behavioral step representing an opportunity to target olfaction and reduce the spread of disease.

The current revolution in genetic engineering, fostered by the application of CRISPR/Cas9, will herald in a wave of new discoveries regarding *Anopheles* olfaction. These new technologies will allow the generation of targeted mutations or genetic knock-ins to capture olfactory expression patterns. In this review, we identified many open questions that we expect will be addressed by applying such new methods. CRISPR/Cas9 has also enabled the development of gene drives methods that can be used to decimate entire mosquito populations (Adolfi et al. 2020; Carballar-Lejarazú et al. 2020; Simoni et al. 2020). The decision to enact such measures requires rigorous research and debate. Gene-drive mechanisms could also be used to target the *Anopheles* olfactory system, and hence alter olfactory host-seeking behaviors.

The world is full of odors, and *Anopheles* mosquitoes, like most insects, have evolved sensitive olfactory systems to turn volatile chemicals into perceptions of the external world. Ultimately, the

mosquito brain combines information from other sensory systems (such as hearing, taste, vision, thermoreception) to make optimal behavioral decisions. In the years to come, investigation of *Anopheles* olfaction will play a central role in the emerging field of mosquito sensory biology.

Authors' note

The *An. gambiae* mosquito (of the *An. gambiae* species complex) can be categorized into an S form (*An. gambiae* ss) and M form (*Anopheles coluzzii*) (della Torre et al. 2001; Coetzee et al. 2013). These 2 forms are morphologically indistinguishable and require molecular techniques for identification (Powell et al. 2014). The M and S forms exhibit a range of hybridization depending on the geographical region examined; for example, in the Cameroon, reproductive isolation is complete (Wondji et al. 2005), whereas in The Gambia, M/S hybrids have been found in frequencies nearing 17% (Caputo et al. 2008). Since molecular methods are required to distinguish M versus S forms, many studies may not necessarily report which *An. gambiae* form (M or S) was under investigation. In this review, the specific form is specified if known (*An. gambiae* s.s. for S form and *Anopheles coluzzii* for M form). If a distinction is not made in the review (*An. gambiae*), either form may have been the species used in the study.

Funding

This work was supported by grants from the Department of Defense to C.J.P. (W81XWH-17-PRMRP), the National Institutes of Health (NIH) to C.J.P. (National Institue of Allergy and Infectious Diseases R01Al137078), the Johns Hopkins Malaria Research Institute Pilot Fund to C.J.P., Natural Science and Engineering Research Council Postdoctoral Fellowship (NSERC-PDF) to J.K.K, and a Johns Hopkins Malaria Research Institute Postdoctoral Fellowship to S.M. We thank the Johns Hopkins Malaria Research Institute and Bloomberg Philanthropies for their support.

Acknowledgments

We thank D. Poinapen for comments on an earlier version of this manuscript.

Conflict of interest

The authors declare no conflict of interest.

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