

University of Nevada, Reno

**Mosquito odor receptor gene targets to alter vector host choice**

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requirements for the degree of Doctor of Philosophy in  
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By

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## ABSTRACT

Olfaction plays an important role in mosquito behaviors, including foraging for nectar and identifying potential hosts for a blood meal. Mosquitoes detect semiochemicals through the use of odor receptors, each of which interact with specific odorants. Differences in feeding behaviors have significant consequences for the vectorial capacity of *Anopheles* mosquitoes. Mosquitoes in the genus *Anopheles* vector the pathogens causing human malaria, which resulted in over 600,000 deaths in 2020 (WHO World malaria report 2021). Yet many species of *Anopheles*, including *An. quadriannulatus*, are competent for malaria but are not significant vectors (Takken & Verhulst, 2012). A defining characteristic of the most important vectors of malaria, which includes the significant urban vector *An. stephensi*, is a proclivity for feeding on human blood. Multiple sensory cues influence mosquito behaviors, and olfaction is particularly important to host preference. Among mosquito chemosensory gene families, odorant receptors (ORs) are receptive to volatile components of sweat, breath, and skin microbiota which mosquitoes use to identify hosts for blood feeding.

The hypothesis that odor receptors with differences in transcriptional abundance between anthropophilic and zoophilic species, male and female mosquitoes, and blood fed and non-fed females mediate differences in mosquito host choice was tested. Using the *Drosophila* empty neuron system, we characterized odorant receptors from two species of *Plasmodium* competent mosquito, one anthropophilic (*An. stephensi*) and the other zoophilic (*An. quadriannulatus*). The odorant receptors were characterized with a panel of structurally diverse odorants, many of which are components of human and animal odors. *Anopheles stephensi* is an important urban malaria vector in the Middle-East, Asia and now Africa. The first functional characterizations of chemosensory genes in this species are presented in this dissertation.

Using RNAseq, we measured chemoreceptor expression patterns in the *An. stephensi* mouthparts, which are important to mosquito host seeking behavior. An odor receptor, *An.*

*stephensi* Or8, which is prominently expressed in the mouthparts was functionally characterized in the empty neuron system. Or8 was found to strongly interact with human sweat odorants, including 1-octen-3-ol and sulcatone. Moreover, capitata peg B neurons on the *An. stephensi* maxillary palps responded to odorants with the same activity pattern identified for Or8 expressing *Drosophila* empty neurons. This work is presented in Chapter 2 of this dissertation.

We characterized several *Anopheles* odorant receptors with expression differences between anthropophilic and zoophilic species, which are upregulated in female and non-blood fed mosquitoes. The odor receptors characterized had diverse activities, which included inhibiting the empty neuron in response to odorants. The diverse activity patterns allow for comparisons with odor receptors previously characterized in other species, provides new insights into the conservation of odor receptor functions between *Anopheles* which diverged ~50 MYA.

One odor receptor from the zoophilic *An. quadriannulatus*, AqOr9, detected aromatic molecules from the original odorant panel. AqOr9 activity was further explored in a reiterative process, of selection for molecules with structural similarities to the most activating odorants, in order to identify environmental sources which activate olfactory receptor neurons expressing this receptor. It was found that the receptor responds most strongly to guaiacols, which to humans have a smoky smell, and which are released upon lignin pyrolysis (Chapter 3).

The final chapter explores a mechanism to alter mosquito host choice. We propose that host choice of vector mosquitoes might be altered by the knockout or substitution of odor receptors sensitive to predominantly human-emitted odors with receptors sensitive to odors emitted in greater abundance by other animals or environmental sources. We develop a construct to replace activity of *An. stephensi* capitata peg B neurons using Cas9 induced double-strand break repair. Ultimately, this research informs an approach to alter vector host choice using gene drives to break the human-mosquito malaria transmission cycle.

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## Chapter 1:

### A review of odor receptor genes as targets to alter mosquito host choice

#### Introduction:

Mosquitoes are the most deadly animals known to man due to blood feeding on humans, which leads to the transmission of pathogens responsible for many diseases. In particular, the Anopheline mosquitoes are vectors for the *Plasmodium* parasites which cause human malaria, which kills more humans than any other vector-borne disease. In 2020, over 600,000 people are estimated to have died of malaria (WHO world malaria report 2020). Of the thousands of known mosquito species, only a few are responsible for vectoring pathogens which cause human disease and death. The species which are important vectors for pathogens which infect humans have shared characteristics: an affinity for feeding on human hosts and the ability to thrive in or to near human habitats. The genetic factors underlying mosquito host seeking and blood feeding behaviors have been studied within the last decade. Chemosensory genes have been implicated as likely candidates for the behavioral differences between species (Carey et al., 2010; DeGennaro et al., 2013; Takken & Verhulst et al., 2013; McBride et al., 2014).

Among the factors involved in mosquito host seeking behaviors, the receptors involved in mosquito olfaction are viable candidates for functional study. Mosquito host seeking and feeding behaviors are largely gated by olfactory cues. A prominent family of genes contributing to mosquito olfaction and related behaviors is the odorant receptor gene family (ORs). The blood feeding behavior of mosquitoes provides an intriguing target for genetic intervention against the spread of vector-borne diseases. ORs offer potential targets for gene editing technology, which could greatly limit the spread of vector-borne diseases. The first part of this review will examine mosquito behavior and other contributing factors that lead to the spread of malaria. The second part of the review will introduce chemosensory receptors that govern mosquito behaviors,

focusing on blood feeding behavior and the odor receptor gene family. Methods for identifying ORs with particular behavioral roles will be discussed. The third review chapter will present an analysis of the use of gene editing technologies to limit vector-borne disease. This review will conclude by examining OR genes as targets for genetic intervention strategies, including gene drives.

## **Part 1: Mosquito characteristics contributing to malaria spread and the role of olfaction in mosquito behavior**

### **1.1 Characteristics of mosquitoes which are important malaria vectors**

Anopheline mosquitoes are responsible for all human malaria transmission. However, most mosquito species, including most Anophelines, do not transmit human malaria. Anophelines inhabit different ecological niches and exhibit a wide variety of host and feeding preferences (Neafsey et al., 2014; Rinker et al., 2013). Factors that contribute to the vectoring of human malaras by mosquitoes include: 1) species immunity or refractoriness to infection with endemic malaria strains, 2) the mosquito habitat and habitat overlap with human populations, and 3) the host preference of the mosquito, anthropophilic or zoophilic (Scott & Takken, 2012). Mosquitoes must also have a life span that supports the development of malaria parasites from acquisition of the gametocyte through transmission of the sporozoite stage to a human host in a subsequent blood meal (Beier, 1998). Other behaviors may contribute to malaria risk. For example oviposition site preference or resting behaviors can also influence the effectiveness of current malaria reduction strategies such as oviposition traps and insecticide treated bednets (Steinhardt et al., 2017).

The vector competence of mosquitoes with respect to malaria parasites varies significantly between species. Only mosquitoes in the genus *Anopheles* may harbor human

malaria parasites, but even within the genus there is substantial variation between species in rates of infection, the parasite load of infected mosquitoes, and the parasite or range of parasites to which mosquitoes are susceptible. For example, in a study of five *Anopheles* malaria vectors collected in the Brazilian Amazon, after experimental infection with *P. vivax* there was observed variation in susceptibility to infection, infection rates between species, and average oocyst load (Rios-Velasquez et al., 2013). Mosquito species also vary by refractoriness to infection with different strains of malaria, as shown by different rates of infection with different malarial strains among species of field-caught mosquitoes (de Arruda et al., 1986; Branquinho et al., 1993). Plasmodium parasites also exhibit differences, such as in mechanisms of oocyst escape, which may be adaptations to the specific host Anopheline environment (Orfano et al., 2016). Even among subspecies, one group can be more susceptible to malaria infection due to genetic factors, as was shown in a study of *An. gambiae* s.s. and *An. colluzzii* (formerly *An. gambiae* S and M) fed on infected blood, in which the *An. gambiae* s.s. developed higher parasite loads (Ndiath et al., 2011). Individual mosquitoes may also be more or less immune to disease due to underlying genetics, which has previously been demonstrated by selecting malaria-resistant strains of mosquitoes (Collins et al., 1986) and the subsequent identification of resistance alleles (Blandin et al., 2009). The mosquito gut microbiota may also influence mosquito susceptibility, as it was previously shown that Enterobacteriaceae abundance was highly elevated in the guts of malaria (*P. falciparum*) infected *An. gambiae* among mosquitoes challenged with *P. falciparum* infection (Boissiere et al., 2012). Oviposition site choice, which is in part guided by olfactory cues (Afify & Galizia, 2014), influences the mosquito gut microbiota composition (Boissiere et al., 2021).

The factors discussed above, immune system, adaptations to habitat, and olfaction provide clues to the genes contributing to mosquito species propensity to transmit malaria. This review will focus primarily on the role of olfaction in mosquito behaviors, and the mosquito chemoreceptors which interact directly with environmental olfactory cues.

### **1.2A *Anopheles gambiae* species complex**

Members of the *Anopheles gambiae* species complex (*gambiae s.s.*, *coluzzii*, *arabiensis*, *melas*, *merus*, *quadriannulatus*, *bwambae*, *amharicus* and *fontenillei*) include the predominant malaria vectors in sub-Saharan Africa, where the species complex is endemic (Neafsey et al., 2015; Thawornwattana et al., 2018; Barrón et al., 2019). Species within the complex are defined by behavioral and habitat differences, and host preferences for blood feeding is an important distinguishing behavioral trait. Species within the complex are each vector competent for *Pl. falciparum*, but only the anthropophilic species, most significantly the subspecies *An. gambiae s.s.*, *An. colluzzi*, and *An. arabiensis*, are important malaria vectors (Takken & Verhulst, 2013). *An. quadriannulatus* and *An. amharicus* are both zoophilic members of the clade which do not spread malaria. Given the close relatedness of *An. quadriannulatus* to significant anthropophilic vectors, it is therefore an ideal reference species for the study of olfactory differences contributing to distinct feeding behaviors (Scott & Takken, 2012; Rinker et al., 2013). Within the *An. gambiae* complex, anthropophilic feeding likely evolved independently multiple times within the last ~500,000 years (Kamali et al., 2012). *Anopheles quadriannulatus* diverged ~500,000 years ago from the *An. gambiae* common ancestor, and ~250 thousand years ago from another anthropophilic and competent vector species, *An. arabiensis* (Thawornwattana et al., 2018). This period of convergent evolution of anthropophily dates prior to the evolution of modern humans, a period when distinct hominin populations roamed the sub-Saharan savannas (Bergstrom et al., 2021).

### **1.2B *Anopheles stephensi***

*Anopheles stephensi*, known as the South Asian malaria mosquito, is, as the name suggests, the predominant urban vector of malaria in India and Pakistan (Jiang et al., 2014). The species range extends into the Middle East and China (Manouchehri et al., 1976; Sinka et al.,

2011). *Anopheles stephensi* is a competent vector for the most deadly species of malaria, *Plasmodium falciparum* (Oakley et al., 2018), as well as the other malarials with the most significant impacts globally on human health, including *P. vivax* and *P. knowlesi* (Hawking et al., 1957; Seyfarth et al., 2019). *An. stephensi* is also a significant vector for plasmodia which cause malaria in other species, such as the murine malaria agents *P. berghei* and *P. yoelii* (Xu and Dong et al. 2005, Esperanca et al., 2018). Historically, *An. gambiae* has been the primary vector of malaria in Africa (esp. sub-Saharan). More recently, *An. stephensi* has been found in Ethiopia and the Horn of Africa, raising further concerns about the spread malaria in these regions attributable to *An. stephensi* (Balkew et al., 2020; Seyfarth et al., 2019).

The habits of *An. stephensi* places this species in close contact with very large human populations and contributes directly to the propensity of the species to spread malaria. Other vectors in rural India, including *An. culicifacies*, are highly competent vectors (Adak et al., 1999), but lack adaptations which permit malaria to spread in an urban environment. *Anopheles stephensi* successfully lays eggs in domestic environments, in open water containers, cisterns, and puddles, and consistently feeds indoors in an urban environment (Sinka et al., 2011). *Anopheles stephensi* found in urban environments exhibit anthropophily and exhibit greater attraction to human odor than the odors of cattle or guinea pigs (Omrani et al., 2010; Sinka et al. 2011). *Anopheles stephensi* in Chennai, India, a city known for a high rate of malaria infection, exhibit a resting preference in cattle sheds over human dwellings. This behavior protects the mosquitoes against traditional application of insecticides and repellents in human dwellings (Thomas et al., 2017). *Anopheles stephensi*, already armed with several adaptations to an urban environment, continues to evolve and expand its range during ongoing global urbanization (Thakare et al., 2022). In conclusion, the habitat preferences, feeding preferences and vector competence of *An. stephensi* combine to make this species a potent vector for malaria in urban Southeast Asia, and more recently in Africa.

### **1.3 Biology of the infective agent, *Plasmodium*, and life stages that present targets to limit vector transmission**

#### **1.3A The malaria life cycle**

*Plasmodium* is an apicomplexan, which is a group of single-celled eukaryotic parasites, many of which are important vertebrate parasites, including *Toxoplasma* and *Cryptosporidium* (Arisue & Hashimoto, 2015). Apicomplexans are alveolates, a diverse group of organisms which includes single-celled predators, photoautotrophs, and parasites (Moore et al., 2008). Parasitism has evolved multiple times within the alveolate clade (Arisue & Hashimoto, 2015). The apicomplexans are derived from a photosynthetic common ancestor (Moore et al., 2008).

*Plasmodium spp.*, the disease causing agents of malaria, are vector-borne parasites dependent upon on mosquito and vertebrate hosts. Mosquitoes of the genus *Anopheles* are responsible for the vectoring of mammalian malaria (Neafsey et al., 2015), which is transmitted during blood feeding when sporozoites exit through the mosquito proboscis via the saliva (Ponnudurai et al., 1991; Beier et al., 1991). In the vertebrate stages of development, *Plasmodium* first infect liver cells. After several rounds of replication within hepatocytes, merozoite stage parasites exit the liver (Menard et al., 2013). Next, the parasites infect erythrocytes and undergo several rounds of proliferation, and at this stage the clinical symptoms of malaria manifest in the host (Menard et al., 2013). During blood stage replication a small fraction of parasites differentiate into gametocytes (Morillo et al., 2022). Mature gametocytes infect the mosquito during a blood feeding event and undergo sexual reproduction within the mosquito midgut (Menard et al., 2013). Within the bloodmeal, gametes produce zygotes, which then differentiate into ookinetes (Beier, 1998). Ookinetes then penetrate into the midgut lumen, and can be detected within 24-30 hours of infection of *Anopheles* with *P. falciparum*, and as few as 8 hours for rodent malaras (Beier, 1998). Ookinetes then develop into oocysts, which undergo a long period of

differentiation into the infective form of the malaria, the sporozoites which migrate through the hemolymph into the mosquito salivary glands (Beier, 1998). Oocyst development provides an intriguing bottleneck in malaria development in the mosquito, requiring at least 10 days to develop into sporozoites (Beier, 1998). Altogether, *Plasmodium* requires about 14 days to fully develop into the infective sporozoite stage once gametocytes have entered the mosquito. After infection of humans, *P. falciparum* requires 3-4 weeks to develop into gametocytes which are transmissible to mosquitoes, while other malarias may take as few as 1 week in rodents and 2 weeks in humans to reach the gametocyte phase (Menard et al., 1998; Morillo et al., 2022).

### **1.3B Vulnerabilities in the malaria host transmission cycle**

The *Plasmodium* host cycle presents several opportunities for limiting both the impact and spread of the disease. Of particular note is the vector-vertebrate interaction. Disease transmission can be disrupted by reducing feeding events. Importantly, malaria may be transmitted by mosquitoes during probing, which occurs prior to feeding and does not commit the mosquito to blood feeding on a particular host (Matsuoka et al., 2002). Probing behavior without blood feeding could therefore increase the number of malaria transmission events.

The most effective methods of malaria control to date have relied upon reducing the vector-vertebrate host interaction. One well studied example is the use of bed nets and screens, which protect against malaria in areas where predominant vectors feed at night and indoors (Huo et al., 2013). In other circumstances, bed nets do not effectively reduce vector-vertebrate interactions. Bed nets do not significantly reduce transmissions from *Anopheles* which habitually bite outdoors and during periods when people are active in the evening or at night (Steinhardt et al., 2017).

Reduction of feeding events has historically been approached indirectly by eliminating or reducing vector population to the largest extent possible, especially through habitat hygiene and

the application insecticides (Gu & Novak, 2005). The extensive period of sporozoite development in the mosquito, known as the extrinsic incubation period, requires 10 or more days (Beier, 1998). The malaria niche is vulnerable to technologies that can artificially reduce the mosquito lifespan. Odorant baited traps are, in theory, a viable strategy to reduce the transmission of vector-borne diseases (Ray, 2015).

Other factors in the malaria host relationships provide other opportunities to reduce the malaria niche. Taxonomic research on malaria has shown that: 1) each individual malaria species has a unique host range (malaria parasites are specialists, not generalists, ecologically), (Borner et al., 2016) 2) *Plasmodium* differ in their abilities to infect different vector species (Joshi et al., 2009), and 3) different species (or strains) of *Anopheles* exhibit differential refractoriness to malaria infection (Mohanty et al., 2018), which can be a general trait or dependent upon the species of parasite. Each of these ecological factors is important to vector competence and the degree to which individual mosquito species and strains contribute to malaria infections in humans.

### **1.3C Barriers to the interspecies transfer of malarias**

Malaria strains exhibit host specificities which presents a barrier to interspecies vertebrate host transfers (Ngoubangoye et al., 2016). Although rare, transfers of vertebrate host-specific malarias have been shown to occur between closely related species, such as between great apes (Ngoubangoye et al., 2016). The two most significant human malaria parasites, *P. vivax* and *P. falciparum*, each originated from separate transfers from great apes to humans, and in the case of *P. falciparum* ancestor, the transfer from gorillas most likely occurred within the last 1 million years (Rougeron et al., 2021). Further research on the host ranges of malaria parasites in avians has shown that *Plasmodium* species exhibit host specificity (Ventim et al., 2012). There is conflicting evidence on the transfer of malaria between humans and bovines

(Franco et al., 2014; Hasyim et al., 2018). Further research is needed on the host specificities of malarias which infect livestock (Albadrani et al., 2021).

#### **1.4 The role of olfaction and odor receptors in mosquito behaviors**

Multiple sensory cues contribute to mosquito host seeking behaviors, including odorants, tastants, visual cues, and heat. Multisensory integration is another important component of mosquito behaviors (van Breugel et al., 2015; Vinauger et al., 2019). *Aedes aegypti* demonstrate an interdependence on CO<sub>2</sub> and heat cues governing host seeking and landing dynamics of female mosquitoes (McMeniman et al., 2014; van Breugel et al., 2015). Visual and odorant cues are also integrated in mosquito host seeking behaviors, with CO<sub>2</sub> gating female *Aedes aegypti* visual tracking of discrete objects (Vinauger et al., 2019). Studies in *Drosophila melanogaster* similarly demonstrate that an attractive odorant enhances the tracking of visual stimuli in dipterans (Frye & Dickenson, 2004; Chow & Frye, 2008).

While the importance of heat and visual cues to mosquito host seeking and biting behaviors cannot be overstated, the primary focus of this review is the olfactory system of mosquitoes. The remainder of this section explores in greater detail the role of olfaction in mosquito host seeking and blood feeding behaviors, and other state dependencies, including age, blood fed status and circadian rhythm which that modulate mosquito behavior via olfaction.

##### **1.4A Organization of the peripheral olfactory system in mosquitoes and *Drosophila***

Insect odor receptor neurons (ORNs) interact with volatile organic compounds (VOCs) via odorant receptors (ORs). Each ORN expresses a single ‘tuning’ OR which has a specific response to VOCs and determines the range of sensory properties of the neuron. The peripheral component of the *D. melanogaster* olfactory system is strikingly similar to the mosquito system, and thus provides a reasonable model for the study of olfaction. In both flies and mosquitoes,

sensilla housing ORNs are most concentrated on the antennae, but chemosensory sensilla can also be found on the mouthparts, wings and legs (Joseph & Carlson, 2015). Expression of *orco*, an odorant receptor co-receptor required for the formation of functional odorant receptors for most insects, can be used to identify the ORNs (Riabinina et al., 2016; Butterwick et al., 2018). The adult *D. melanogaster* antennae houses about 1,000 ORNs with sensory properties determined by the specific expression of about 4 dozen ORs (Vosshall et al., 2000). The antennae of female *An. gambiae* mosquitoes houses an estimated 1,250 ORNs (Riabinina et al., 2016). The olfactory system of *An. gambiae* exhibits a pronounced sexual dimorphism not seen in *D. melanogaster*. Female mosquitoes have ~2.5 times the number of antennal ORNs of males and an antennal lobe which is about two times larger (Riabinina et al., 2016). This dimorphism could be attributed to an increased reliance on the olfactory system for informing the female specific behaviors of host seeking and ovipositioning.

ORN axons in both *Anopheles* and *Drosophila* project to glomeruli in the antennal lobe. Each individual glomeruli consists of axonal projections from ORNs which express the same tuning odorant receptor gene (Fishilevich & Vosshall, 2005). The *D. melanogaster* antennal lobe contains 47 distinct glomeruli, which is equivalent to the number of expressed odorant receptor genes in imagos (Fishilevich & Vosshall, 2005). The *An. gambiae* antennal lobe is more complex. It contains ~70 glomeruli, only half of which are innervated by ORNs. The other half are likely innervated by sensory neurons that express other types of chemosensory receptors (Riabinina et al., 2015). Adult *An. gambiae* have fewer antennal lobe glomeruli innervated by distinct ORN populations (33) than *D. melanogaster*, although they have a larger genomic repertoire of ORs (Neafsey et al., 2014). It is unknown how the complexity of the *An. stephensi* antennal lobe compares, although it is notable that the *An. stephensi* genome has fewer OR genes (59) than *An. gambiae* (76) (Rinker et al., 2013a; Speth et al., 2021). Interneurons facilitate feedback signaling between glomeruli within the antennal lobe, and glomerular signals are carried

to the mushroom body and lateral horn via projection neurons (Su et al., 2009). It is possible that other mosquito odorant receptors are more distributed throughout sensory hairs on the legs, wings and ovipositor than is the case for *D. melanogaster*.

#### **1.4B The role of olfaction and odor receptors in mosquito foraging behaviors**

Female and male mosquitoes feed on nectar, which increases the lifespan, but not fecundity of *Anopheles* and *Aedes* mosquitoes (Foster, 1995; Manda et al., 2007). In fact, members of one mosquito group, *Toxorhynchites spp.*, do not take blood meals, and the adults of this clade rely solely upon nectar and sap for survival (Steffan & Evenhuis, 1981). While visual cues play a role in the identifying of preferred plants (Dieng et al. 2018), mosquitoes are able to locate favored nectar sources via olfactory cues alone (Lahondere et al., 2021). Mosquito attraction to floral scents is mediated by a balance of attractive floral odorants, including nonanals, and repulsive odorants, which include lilac aldehydes (Lahondere et al., 2021). *An. gambiae* exhibit specificity in attraction to plant nectar sources, and attractive plants often exhibit little overlap in the presentation of most abundant floral volatiles (Nikbakhtzadeh et al., 2014). *An. gambiae* survival and ultimate fecundity are increased via feeding on more attractive nectar sources (Manda et al., 2007). This could provide a source of positive selection on mosquito ORs which detect floral scent markers of nutrition. Although *Aedes* mosquitoes occupy different habitats than *Anopheles* mosquitoes and must rely upon different native nectar sources, each exhibit similar attraction to odorant blends derived from the orchid *Plantathara obtusata* (Lahondere et al., 2021), which suggests that semiochemical recognition of quality nectar sources is at least partially conserved between the Anophelinae and Culicinae. Olfaction also plays a role in mosquito ovipositioning. Gravid female *Ae. aegypti* are attracted to the odorant geosmin, which is produced by both algae and photosynthesizing aquatic bacteria, and a preference to geosmin treated water is ablated in orco mutants (Melo et al., 2020). Interestingly, *Ae. aegypti*

larvae also exhibit attraction to geosmin (Melo et al., 2020), which could be used to localize food sources.

Olfaction is important to dipteran larval foraging behaviors. In comparison to adult olfactory systems, larval olfactory systems are numerically simpler and non-redundant (Mathew et al., 2013). *Drosophila melanogaster* larvae express 25 distinct OR genes, 21 of which are functional (Mathew et al., 2013), and half of which are larval specific (Joseph & Carlson, 2015). Larval ORNs innervate the larval dorsal organs, anterior and bilaterally symmetrical dome-like structures. Each unique larval OR is expressed in a single ORN (Kreher et al., 2008). Despite the relative simplicity of the larval olfactory system, *D. melanogaster* larvae are able to distinguish amongst a large number of structurally diverse odorants (Mathew et al., 2013). In turn, larvae have different behavioral responses are largely dependent upon the activity of just a few receptors expressed in an equivalently small number of neurons (Kreher et al., 2008). Odorants which activate a single larval ORN are sufficient to mediate chemotaxis (Mathew et al., 2013; Dweck et al. 2015).

Odorant driven behavior in mosquito larvae is currently a murkier picture. Mosquito larvae have bilaterally symmetrical, relatively simple antennae, which are developmentally related to the adult structure (Riabinina et al., 2015). Mosquito larvae have only about 10 ORNs per antennae (Riabinina et al., 2015). Mosquito larvae are aquatic, and given that the insect ORs are an adaptation to terrestrial life (Brand et al. 2018), the other major chemosensory gene families, the ionotropic receptors (IRs) and gustatory receptors (GRs), could play a relatively greater role in mosquito larval chemotaxis and navigational functions than in *D. melanogaster*. In spite of the reduced combinatorial complexity of the mosquito larval olfactory system, larval ORNs of both *Aedes* and *Anopheles* are able to detect many odorants (Bui et al., 2019; Sun et al. 2021), some of which are attractive or repellent. In addition, in *orco* mutant *Anopheles* larvae,

attraction to baker's yeast, a food source, is impaired (Sun et al., 2021). However, it remains to be determined which specific mosquito larval ORs are responsible for detecting of food odors.

#### **1.4C The role of olfaction and odor receptors in mosquito host seeking and blood feeding**

Olfactory senses are critical to mosquito host seeking and landing behaviors. The role of CO<sub>2</sub> sensing and the capitata peg A neurons (cPa) neurons expressing the CO<sub>2</sub> receptor in mosquitoes has been particularly well studied in the context of host seeking behavior in both *Aedes* and *Anopheles*. Chemical inhibition of the CO<sub>2</sub> sensing neuron greatly reduces the landing behavior of *Aedes aegypti* females on a human hand (Tauxe et al., 2013). *Aedes aegypti* females exhibit a preference towards traps treated with activating compounds for cPa neurons, with the stimuli eliciting the largest increases in cPa neuron activity resulting in the greatest attraction (Tauxe et al., 2013). However, mosquito species and genus are differentially attracted to CO<sub>2</sub>. Behavioral studies with *An. stephensi*, *An. gambiae* and *An. quadriannulatus* show that while *An. stephensi* and *An. quadriannulatus* exhibited a slight attraction to CO<sub>2</sub> alone, *An. gambiae* did not. In these same studies, all *Anopheles* species exhibited greater attraction to a combination of an odor with CO<sub>2</sub>, but each species was most attracted to a different odor used in combination (Takken et al., 1997; Pates et al., 2005). CO<sub>2</sub> is repellent to mosquitoes at higher concentrations, but repellent concentrations are vary by species. In general, the role of CO<sub>2</sub> in attraction and host seeking behaviors of Anophelines is quite variable with respect to CO<sub>2</sub> concentration dependence and odorant interdependence (Constantini et al., 1996; Takken et al., 1997; Pates et al., 2005).

CO<sub>2</sub> plays a well resolved role in gating upwind flight during host seeking in both the *Aedes* and *Culex* genera (Vinauger et al., 2019). CO<sub>2</sub> sensitizes the mosquitoes to both odor and visual cues (Vinauger et al., 2019; Dekker et al., 2005). The CO<sub>2</sub> sensing unit, the capitata peg on the maxillary palp, including a 1-octen-3-ol sensing neuron, is also conserved in *Anopheles* mosquitoes, and is very likely an ancestral trait of Culicidae (Lu et al., 2007; Bohbot et al., 2013;

Speth et al., 2021). Other factors must modulate sensitivity to CO<sub>2</sub> and behavioral differences between species. While CO<sub>2</sub> sensing is the best studied example of the role a single odorant plays in mosquito behavior, other components of odors determine the host preferences of a mosquito species.

*Anopheles spp.* exhibit variable attraction to odors collected from different animal sources. Different species showing more or less attraction dependent upon the odor source (Constantini et al. 1998). Some mosquitoes are more generalist and do not exhibit strong preferences to odors from different host sources. Mosquito attraction to specific odorants can be dependent on the genus, individual species, age, and other states of the mosquito. To list just a few known examples which demonstrate these principles of olfaction dependence, *Ae. aegypti* exhibit a strong attraction to lactic acid, but *Anopheles* are only weakly attracted to this odorant (Raji et al., 2019; McBride, 2016). 6 day old female *Ae. aegypti* are more strongly attracted to a combination 1-octen-3-ol and CO<sub>2</sub> than 10 day old females, yet 1 day old females are not attracted to these odors (Bohbot et al., 2013). 4 day old *An. colluzzii* adult females exhibit attraction to human odor blends and 1 day post emergence females are deterred by the same odor blends (Omondi et al., 2019).

Malaria infection also induces an increase in attraction to vertebrate hosts including humans, mice, and avians. Birds infected with the avian malaria parasite *Plasmodium relictum* were more attractive to *Culex pipiens*, although the sensory cues responsible for this increased attraction were not established (Cornet et al., 2013). Human children harboring gametocytes of *P. falciparum* are more attractive to *An. gambiae* (Lacroix et al., 2005). Malaria infection in children results in increased emanation of several volatiles, including 2-octanone, nonanal and heptanal (Robinson et al., 2018). *Anopheles coluzzii* are more attracted to human odor blends supplemented with odorants which constitute a greater density of the human skin odor space during the gametocyte stage of *Plasmodium* infection (Robinson et al., 2018). While the heat

from malaria-induced fever is potentially an important factor in increased host attractiveness, *An. stephensi* prefer malaria-infected mice and humans which is specifically attributable to odor (de Moraes et al., 2014). In mice, infection with the murine malarial agent *P. chabaudii* resulted in increased emission of several volatiles, including 2-phenylethanol and 3-methylbutanoic acid and tridecane. *Anopheles stephensi* exhibit a greater attraction to both infected mice and mouse odor supplemented with volatiles with increased emission during *Plasmodium* infection, versus healthy mice and non-supplemented odors from healthy mice, respectively (de Moraes et al., 2014).

The skin microbiome is responsible for the production of many vertebrate animal odors. Both the genetic composition and the growth stage of bacteria result in the production of different blends of volatile organic compounds (VOCs), and mosquitoes are differentially attracted to these odor blends (Verhulst et al., 2010). *Anopheles gambiae* is more attracted to odors produced by microbes in the stationary phase than the growth phase, during which different metabolites are produced (Verhulst et al., 2010). The mosquitoes are also more attracted to individual odorants characteristic of particular bacterial species (Verhulst et al. 2010).

Mosquitoes also exhibit state-dependent attractive behavior in response to odors. Blood fed female mosquitoes do not host seek and exhibit decreased olfactory responses. Female mosquitoes exhibit transcriptional changes in response to blood feeding across multiple genera, which are presumably important for the observed changes in olfactory driven behavior (Fox et al., 2001; Taparia et al., 2007; Rinker et al., 2013). While there is surprisingly little behavioral evidence for blood feeding induced changes in female mosquito attraction to particular odorants, the responsivity of a subset of *An. gambiae* ORNs decreases between pre- and post- blood feeding in response to some odorants (Qiu et al., 2006).

Examining the OR gene transcripts, which vary depending upon state-induced changes, could provide clues to which odorant receptors are involved in host seeking and ovipositioning behaviors. For example, *An. gambiae* females have decreased transcript abundance of Or1 (a

tuning OR) 12 hours after blood feeding (Fox et al., 2001). Blood fed female *An. stephensi* have fewer transcripts of the odor receptor co-receptor gene Orco and a tuning OR, Or8, and of the CO<sub>2</sub> receptor genes Gr22, Gr23 and Gr24 (Speth et al., 2021). *Anopheles gambiae* females exhibit changes in chemosensory gene expression after blood feeding, resulting in differences in odorant receptivity (Rinker et al., 2013b). Male and female mosquitoes also exhibit significant differences in transcript abundance of chemosensory genes, including ORs, IRs and GRs (Pitts et al., 2011). Many sex-dependent differences in transcription of chemosensory genes are consistent between species (Athrey et al., 2020). While mosquitoes do not exhibit significant sexual dimorphism in the total number of neurons in brain tissue, female mosquitoes have over twice the number of olfactory neurons as males in the antennae, and ORNs in female mosquitoes inhabit many more antennal segments (Raji & Potter, 2021; Riabinina et al., 2016). This information suggests an outsized importance of the olfactory sensory modality in female mosquitoes, which could play an important role in sex-dependent behaviors, including host seeking.

Mosquito behavior is also dependent upon time and light cycles. Light-dark cycles influence female *Anopheles* flight activities and host seeking behavior, with female *An. gambiae* and *An. stephensi* each exhibiting the greatest activity at the beginning and end of the light phase (Rowland, 1989; Jones and Gubbins, 1978). Circadian rhythm genes regulate mosquito mating behaviors including male swarming (Wang et al., 2021). Circadian clock genes are in turn regulated by light and temperature cues (Wang et al., 2021). Male pheromone synthesis exhibits rhythmicity and is regulated by clock genes (Wang et al., 2021). Female blood-feeding behavior is under control of clock genes and can be altered by light exposure (Das & Dimopoulos 2008). Olfaction also exhibits dependence upon light-dark cycles. *Anopheles gambiae* OBP protein abundance is cyclical over a 24 hour period and the antennal response to hydrophobic odorants is also dependent upon the light-dark cycle (Rund et al., 2013).

## **Part 2: Anopheles chemosensory receptors, and the identification of odor receptors contributing to host preference**

### **2.1 Introduction to chemosensory genes in insects: families and functions**

#### **2.1A The evolution of insect chemosensory receptors**

Insect sensing of semiochemicals is mediated predominantly by three different types of receptors: the ORs, ionotropic receptors (IRs) and the gustatory receptors (GRs) (Neafsey et al., 2014; Pitts et al. 2017). Insect chemosensory receptors are not evolutionarily related to G-protein coupled receptors (GPCRs) as are vertebrate ORs (Niimura & Nei, 2005). Insect ORs instead function as gated ion channels which can directly depolarize the neuron transmembrane potential (Mombaerts 2004, Brunet et al. 1996, Butterwick et al. 2018; del Marmol et al., 2021). The GR family in turn is derived from an ancient metazoan family of GR-Like genes which is present in cnidarians and protostomes but absent from vertebrates (Robertson 2015; Saina et al., 2015). The chemosensory function of GR-Like proteins was likely acquired during protostome evolution, as the GR-Like proteins studied in cnidarians and echinoderms play a developmental role (Saina et al., 2015). The insect OR gene family evolved independently within insects as a novel adaptation to terrestrial life and is not present in other hexapod or arthropod genomes (Brand et al. 2018). The insect OR genes are derived from within the GR gene family, which is present in all arthropods (Robertson, 2015). The IR genes evolved independently from the GR and OR superfamily, from an ionotropic glutamate receptor gene, and are expressed in the chemosensory appendages of protostomes which diverged from insects 550-850 million years ago (Croset et al., 2010).

### **2.1B Chemosensory receptor functions**

Each type of insect chemosensory receptor has distinct sensory properties. IRs respond to a narrow range of odorants, including amines, which are not detected by either mosquito or *D. melanogaster* ORs, and carboxylic acids, which are sparsely detected by *D. melanogaster* ORs and are not detected by mosquito ORs (Hallem et al., 2006; Carey et al., 2010 ;Pitts et al. 2017). IRs perform other diverse sensory functions including thermosensation and hygrosensation (Knecht et al., 2016; Ni et al. 2016).

The ORs detect a diverse range of volatile organic compounds which includes but is not limited to: alcohols, aldehydes and ketones, and heterocyclic and aromatic molecules (Hallem et al., 2006; Carey et al., 2010; Mathew et al., 2013). Insect ORs are also important for the detection of pheromones, including mating pheromones, which has been well documented in moths (Sakurai et al.; 2011).

GRs are believed to function primarily as contact sensors of tastants (Ishimoto & Tanimura 2004; Chen & Dahanukar, 2020). The function of neurons expressing GRs in detecting bitter tastants is particularly well studied (Weiss et al. 2011; Ling et al. 2014). Many GRs detect sugars (Jiao et al., 2008) and salts, and a single GRN plays a role in the detection of both salts and sugars (Hiroi et al., 2004). Different GRs respond to distinct concentration ranges of salts and sugars (Fujishiro et al., 1984). GRs also have non-contact sensory functions, including the long-range detection of CO<sub>2</sub> (Kwon et al., 2007; Tauxe et al., 2013).

### **2.1C Odorant binding proteins**

Odorant binding proteins are non-receptor proteins which modulate sensitivity to odors. OBPs are among the most highly expressed proteins in insect sensory tissues, including the antennae and mouthparts. They are secreted into the sensillar lymph of olfactory sensilla (Galindo & Smith, 2001). OBPs in the lymph may bind odors and control response kinetics of odor

interactions with the ORs on the ORN dendrites through odor trafficking. This OBP function is best studied for a subfamily of lepidopteran pheromone binding proteins (Vogt et al. 2015). OBPs have been shown to play a role in attractive behavior to plant odors as well as in oviposition site preference in *D. sechellia* (Matsuo et al. 2007). However, many OBPs which are abundantly expressed in *D. melanogaster* sensilla do not modulate odorant sensitivity (Larter et al. 2016, Xiao et al. 2019). In mosquitoes, OBPs may modulate sensitivity to odors which are important to host seeking behavior. *An. gambiae* OBP (OBP1) exhibits increased transcript abundance in the heads of non-blood fed females and modulates indole sensitivity (Biessmann et al., 2010). However, the functional roles of most mosquito OBPs remain undetermined.

## **2.2 Odor receptors and methods for functional characterization**

### **2.2A Functional chemosensory receptor structures**

While each of these receptor families contributes to insect navigational and feeding behaviors, ORs have been the most comprehensively studied to date. This is due in large part due to the structural simplicity of the two-part orco- tuning OR interaction. Olfactory receptors bind odors and initiate action potentials within the neurons in which they are expressed (Joseph & Carlson, 2015). Individual odor receptor neurons (ORNs) generally express a single odor receptor gene which is responsible for the respective neurons' sensory tuning. To initiate an action potential, a conserved odor receptor coreceptor (orco) pairs with an individual tuning OR in a neuron to form a functioning ligand-gated ion channel (Butterwick et al., 2018). The functioning odor receptor unit is a heterotetramer consisting of tuning-OR and orco subunits (Butterwick et al., 2018). The ORNs of orco mutant mosquitoes have both decreased background electrophysiological activity and greatly reduced responses to odorants (Sun et al., 2020). Many trichoid sensilla, an especially abundant morphological class of sensilla on the mosquito

antennae, exhibit orco-dependent reductions in odorant response (McIver & Siemicki, 1979; Pitts & Zwiebel, 2006; Sun et al., 2020). Orco mutants also have a substantially decreased preference to human versus animal odors, demonstrating the importance of functional ORs to mosquito feeding preferences (Degennaro et al., 2013).

GRs and IRs require more complex pairings of receptors to form functional ligand-gated ion channels than ORs. As a result *D. melanogaster* and mosquito GRs and IRs have been studied less comprehensively. There are three potential co-receptor subunits in the IR gene family, IR8a, IR25a and IR76b, which are broadly expressed in *D. melanogaster* and are highly conserved across Diptera. In *D. melanogaster*, the hygrosensation and thermosensation receptor complexes consist of three IR subunits, of which two are conserved (IR93a and IR25a), and a third distinct IR (IR21a or IR40a for hygrosensation or thermosensation, respectively) confers the unique sensory properties (Knecht et al., 2016). The *Anopheles gambiae* IRs which detect amines and carboxylic acids are composed of two co-receptor IRs, IR25a and IR76b, in combination with two IR41a subunits, or a dimeric combination of the co-receptor IR8a in combination with the tuning IR75k (Pitts et al., 2017). Many IR functions have not yet been identified. This is due to both the difficulty of identifying the correct IR co-receptor pairings for tuning IRs, as well as the functional diversity of the IRs. Transcriptome data may provide valuable clues for further functional characterization of orphan IRs. For example, the IR co-receptors IR25a and IR76b (but not IR8a) are expressed in the mouthparts of *An. stephensi* and the labial palp of *An. coluzzii* along with a reduced set of candidate IR tuning genes (Saveer et al., 2018; Speth et al., 2021).

GR rules for forming functional ligand-gated ion channels may be even more complex than for the IRs. The functional CO<sub>2</sub> receptor consists of subunits of three different GR proteins encoded by the Gr22, Gr23, and Gr24 genes in mosquitoes, and is comprised of a heteromer of Gr21a and Gr63a gene products in *D. melanogaster* (Tauxe et al., 2013; Kwon et al. 2007). Many fly sugar sensors are composed of two distinct GRs, and in some cases the same GR is a

functional component of distinct sugar sensing units (Liu & Wu et al, 2020; Jiao et al., 2008). In the case of bitter sensing neurons in *D. melanogaster*, it is common for several GRs (sometimes more than 10) to be expressed in the same gustatory receptor (GRN) (Weiss et al., 2011; Ling et al., 2014). This increased combinatorial complexity greatly increases the difficulty of determining the individual components of the functional GR multimers.

Insect ORs are unrelated to the vertebrate GPCR family of odorant receptors, and have an inverted orientation relative to GPCRs, with the N-terminal domain intracellular facing while the C-terminal is extracellular (Butterwick et al., 2018). Extracellular loops 1 and 3 are both very short. In a previous structure-function study of an insect OR, mutations in the second extracellular loop (EC loop 2), between the transmembrane domains 3 and 4, resulted in the greatest differences in structure activity relationship (Hughes et al., 2014). In some cases, single amino acid changes in EC loop 2 were sufficient to cause a shift in odorant induced OR activity (Hughes et al., 2014). Mutations in transmembrane domains 5-7, which are part of the anchor domain that interacts directly with orco, are also sufficient to alter odorant induced OR activity (Hughes et al., 2014; Butterwick et al., 2018). Given the topology of insect ORs, EC loop 2 is the most likely odorant binding domain. The structure of an archaeognathan odorant receptor from *Machilis hrabei* has recently been determined, and the odorant binding site for this receptor is located in the EC loop 2 domain (del Marmol et al., 2021).

## **2.2B Methods for functional characterization of odor receptors**

Many insect ORs can be characterized in heterologous expression systems co-expressing the orco co-receptor. This is a clear advantage for receptor deorphanization relative to the GRs and IRs, for which the co-receptor pairs must first be discovered. Potential pairings of IRs and GRs might be inferred from co-expression analysis of sensilla, or transcriptome data from different insect chemosensory organs. *D. melanogaster* GRs have been localized to individual

sensilla using Gal4-UAS driven fluorescent reporters, which reveals complex groupings of GRs (Weiss et al., 2011; Ling et al., 2014). ORs have been extensively mapped on the *D. melanogaster* antennae using a combination of antisense labelled RNA probes, and through electrophysiological characterization of ORs in a heterologous expression system with response mapping to individual sensilla types. Each of these methods confirms the simple pairings of orco- and tuning ORs within ORNs (Vosshall et al., 1999; Dobritsa et al., 2003; Hallem et al., 2004).

Spatial mapping of mosquito ORs on the antennae is largely unknown, although many *An. gambiae* ORs have been functionally characterized (Carey et al., 2010). ORNs have been located on the mosquito antennae using orco promoter driven fluorescent labelling, and show sexual dimorphism in distribution, with ORNs located on the 12 distal segments of the female antennae and the 2 distal most segments of the male antennae (Riabinina et al., 2016). However, the tuning ORs responsible for ORN specificity remain largely unmapped. This represents a significant gap in the understanding olfactory driven behaviors of mosquitoes, as different expression patterns of the same OR could contribute to behavioral differences between species.

The two most commonly used systems to study insect OR function are xenopus oocytes and the *D. melanogaster* empty neuron system (Wetzel et al., 2001; Dobritsa et al., 2003). Within either of these systems, tuning OR function can be characterized while expressed in the presence of a compatible odor receptor co-receptor (Wetzel et al., 2001; Dobritsa et al., 2003). Orco is a highly conserved chemosensory receptor, and the *D. melanogaster* orco has proven to be compatible with tuning ORs from diverse dipterans, including mosquitoes and tsetse flies (Carey et al., 2010; Chahda et al., 2019). The *Drosophila* empty neuron system is also compatible with ORs from other insect orders, including Hymenoptera (Slone et al., 2017). The empty neuron system has several advantages in comparison to *Xenopus* oocytes. Lack of endogenous activity in *Xenopus* oocytes and other cells used with patch clamp electrophysiology make them unavailable to characterize repressive response to stimulants. Studies in the *D. melanogaster* empty neuron

have shown that several ORs induce inhibitory responses to odorants (Hallem et al., 2006; Carey et al., 2010; Mathew et al., 2013). *Xenopus* oocytes also lack other components of the ORN environment, such as OBPs, which can solubilize odorants (Galindo & Smith, 2001).

### **2.3 Identifying ORs contributing to specific behaviors, with an emphasis on host seeking behavior**

Electrophysiological study of ORs can be used to inform hypotheses about the ecological function of OR genes. Odorants are associated with different food sources, locations, or threats in the mosquito environment. Some odorants are components of human or animal odor spaces and may be important for host identification by blood seeking female mosquitoes. For example, 1-octen-3-ol is a VOC which is a component of human sweat odor and cattle breath (Bernier et al., 2000; Hall et al., 1984). 1-octen-3-ol is detected with great sensitivity by an ORN housed in the capitata peg on the mosquito maxillary palps, and this function is well conserved between different species of blood feeding mosquitoes (Lu et al., 2007; Bohbot et al., 2013; Speth et al., 2021). Interestingly, all sensilla on the tsetse fly (*Glossina morsitans*) antennae harbor ORNs that exhibit excitation in response to 1-octen-3-ol (Soni et al., 2019). These findings suggest that 1-octen-3-ol could be an important odor for host recognition for mosquitoes and other insects that feed on vertebrate blood.

*Anopheles gambiae* ORs are responsive to a wide variety of odorants, including human odors (Carey et al., 2010; Wang et al., 2010). Genes likely to be involved in host seeking can be inferred from the sensory property of the ORs by identifying the genes which respond most sensitively to odor components of human emanations. However, it is unclear how well OR functions are conserved between species. *Anopheles stephensi* and *An. gambiae* diverged ~50 MYA (Neafsey et al., 2015). Within the genus *Anopheles*, the odor receptor gene family has one

of the highest evolutionary rates of change, with strong evidence of positive selection within the OR family (Neafsey et al., 2015).

Gene gain and loss events could contribute to species-specific differences of olfactory driven behaviors of *Anopheles*. OR gene repertoires are largely shared between the species. Most Anophelens have about 60 OR genes within their olfactory gene repertoire, which is predicted to be the number of OR genes of the most recent common ancestor. Similarly, the number of gene gain and loss events is low within the OR gene family (Neafsey et al., 2015). However, the few gene gain and loss events could provide valuable clues to the ORs which mediate host seeking.

Changes in gene expression can also modulate behavioral differences. For instance, an urban strain of *Ae. aegypti* (*Aedes aegypti aegypti*) specialized to blood feed on humans, has elevated expression of an odor receptor with mutations which confers heightened sensitivity to a human sweat volatile, sulcatone, in comparison to more generalist forest strains (McBride et al., 2014).

A high-throughput approach to identify OR gene function and significance is to utilize transcriptome data. Given that only female mosquitoes blood-feed, that blood-fed females are refractory to feeding behavior, and that in the wild Anopheline feeding occurs in accordance with a particular circadian rhythm, transcriptome data from mosquitoes of either sex or in different states could be used to identify ORs which are expressed more highly in females in a 'primed' state for bloodfeeding (Pitts et al., 2011; Rinker et al., 2013b; Athrey et al., 2020). This approach not only identifies which OR genes are state-dependently expressed in female mosquitoes, but can also show which OR gene transcripts are most prolific, and therefore may be expressed in a higher proportion of all ORNs.

### **Part 3: Shortcomings in current approaches to mitigate malaria and a role for new gene editing technologies in eliminating malaria transmission**

### **3.1 Why do we need new strategies to combat human malaria?**

In 2019, over 600,000 people are estimated to have died of malaria (WHO world malaria report 2021). Despite the use of multiple interventions to reduce mosquito habitat, protect people from infection using insecticide treated bed nets, and the continued development of antimalarial therapeutics, decreases in malaria deaths and infections have plateaued over recent years. In 24 countries cases have increased since 2015 (World Malaria Report 2021). Hospitalizations and mortalities caused by malaria are more common in children and are associated with comorbidities which increase health costs (Watts et al., 2021). Total rates of malaria induced hospitalization are unknown. In countries with a high burden of malaria, infection can be very common in adults. For example, in Ethiopia about 1 in 7 have contracted malaria as adults (Kendie et al., 2021). Health care costs total in the hundreds of millions of dollars a year for recurrent malaria caused by *P. vivax* (Devin et al., 2021).

Many of the most common interventions, such as treatment with antimalarials, either lack robustness or are subject to disruptions resulting in tens of thousands more deaths per year. The COVID-19 pandemic contributed to an increase of over 10 million malaria cases and tens of thousands more malaria deaths from 2019 to 2020, highlighting a lack of robustness of the most widely applied malaria reduction strategies, which are vulnerable to supply chain disruptions (World Malaria Report 2021). Antimalarial drug resistance is also a recurring and expanding problem, putting the development and discovery of new drugs on a treadmill to outpace the evolution of drug resistance. Malaria has previously evolved resistance to chloroquinines and hydroxyquinone, and artemisinin resistance has more recently emerged (Neafsey, 2013; Payne, 1987; Balikagala et al., 2021). Moreover, artemisinin resistance has developed independently in both Southeast Asia and Africa and is becoming more widespread (Mok et al., 2015; Balikagala

et al., 2021), illustrating the unsustainability of current strategies dependent upon treating malarial infections.

Despite decades of research on malaria vaccines, little progress has been made, and the protection conferred by malaria vaccines is strain specific. While the World Health Organization approved a malaria vaccine for the first time in October 2021, the vaccine lacks efficacy, requires four shots, and immunity wanes rapidly (Vogel, 2021). Recently, a more effective vaccination regimen using attenuated parasites effectively induced immunity (Mwakinwe et al., 2021). However, this method requires concomitant treatment with antimalarials to prevent breakthrough parasitaemia (Mwakinwe et al., 2021). The technology for the recently approved malaria vaccine was developed in the 1980's, and more innovative vaccines are not likely to be approved for widespread use by the Global Health Organization any time soon.

The development of resistance against pyrethroid insecticides within *Anopheles* threatens malaria prevention. Insecticide resistant phenotypes can spread rapidly within *Anopheles* populations (Miles et al., 2017). The geographic expansion of resistance alleles is another problem which adds to the urgency of evaluating more robust control approaches (Miles et al., 2017).

Climate change adds another pressing need for the development of novel strategies to prevent the spread of mosquito vector-borne disease. *Aedes* can undergo diapause, and lay desiccation-tolerant eggs, which, along with adaptations to container breeding, has enabled *Ae. aegypti* and *Ae. albopictus* to rapidly colonize North America after importation from the Old World (Kraemer et al., 2019). An analysis of recent range expansions of *Aedes* and models of future climate and urban development scenarios predicts an increased risk for *Aedes*-borne arbovirus infection on all continents except Antarctica by 2050 (Kraemer et al., 2019). While some *Anopheles* are found in temperate climates, the species which transmit human malarias are restricted to tropical and subtropical environments (Neafsey et al., 2015). *Anopheles* development

depends on high temperature and rainfall (Caminade et al. 2014). Predictions of the impacts of climate change on malaria distribution are quite variable, however it is certain that the distribution of malaria will change globally in response to climate change and urbanization (Caminade et al., 2014; Colon-Gonzalez et al., 2021; Chemison et al., 2021).

### **3.2 Current genetic intervention strategies to prevent vector-borne disease**

Currently the most widely utilized methods to limit malaria spread rely upon a combination of hygienic approaches to reduce or eliminate mosquito habitat (i.e. standing water) and the use of pesticides. Pesticide use is known to have adverse effects on biodiversity, especially of invertebrates (Beketov 2013). The synthetic pyrethroids applied to control mosquito populations can be particularly damaging to nontarget aquatic insects and other aquatic invertebrates (Mian & Mulla 1989). These nontarget effects of widely used pesticides have measurable impacts on biodiversity where they are applied (Beketov 2013, Hua & Relyea, 2019) and can cascade to reduce populations of animals at higher trophic levels (Hua & Relyea 2019). For many widely used pesticides, the full range of environmental impacts only become known decades after their first uses, as was the case for DDT. The use of insecticides for vector control remains ubiquitous. Alternative approaches which could both limit vector-borne disease spread and decrease off-target impacts have become more technologically feasible within the last decade. Gene editing technology in particular is promising, because its successful use could mitigate the adverse nontarget effects of pesticide use.

An intervention that has very recently gained traction in the United States for mosquito control is the use of the sterile insect technique. The sterile insect technique involves releasing large numbers of sterilized males to compete with natural populations of males, preventing successful mating. This technique was used to eradication of the primary screwworm fly from North America, starting in the 1950s, through the release of hundreds of millions of male

screwworm flies sterilized by radiation. In theory, this technique may be adopted to any insect species in which the females mate only once, or few times (and the males mate repeatedly), which applies to many species of mosquitoes, including important disease vectors, such as *Ae. aegypti* (Massonnet-Bruneel et al., 2013). Technical challenges, such as reduced male mosquito mating resilience after radiation sterilization treatment, initially reduced viability of the sterile insect technique as a mosquito management tactic. The development of systems to introduce genetic sterility, with minimal fitness costs, has made the method into a potential panacea for mosquito-borne disease.

In a field trial for the application of sterile insect technique to mosquito control in the Florida Keys in 2021, Oxitec LTD released genetically modified male mosquitoes which transmit a gene resulting in lethality in the pupal stage (Waltz, 2021; Phuc et al., 2007). Field trials were previously attempted in Brazil, and were initially successful at significantly reducing *Ae. aegypti* populations in urban environments (Carvalho et al., 2015). A combination of genetic modification with the sterile insect technique provides several advantages over the earlier methods, including decreasing fitness cost, and the potential to bias lethal transgene expression to different sexes and towards specific developmental stages through promoter selection (Massonnet-Bruneel et al., 2013; Parbhakargouda et al., 2014; Phuc et al., 2007).

Gene drives are genetic elements which are transmitted to offspring in a biased manner which increases their own rate of inheritance, and can naturally spread through populations despite imposing a fitness cost (Burt & Trivers, 2006). Early attempts at gene drives in *Anopheles* mosquitoes showed great promise for the development of mosquito population suppression mechanisms. The earliest gene drive implemented in mosquitoes utilized a mitochondrial endonuclease, I-SceI, as a proof-of-principle (Windbichler et al., 2011). Cas9-based homing endonuclease gene (HEG) drives have much greater functionality. For the first demonstration of a Cas9-based HEG drive in mosquitoes, a gene drive carrying two antimalaria

effector genes was integrated into the *An. stephensi* genome (Gantz et al., 2015). In this case, the homing construct was an impressive 17kb, demonstrating the potential of Cas9 based gene drives to efficiently copy multiple genes under the control of unique promoters in a single construct (Gantz et al., 2015). Interestingly, this *An. stephensi* gene drive had a higher frequency of transmission when paternally transmitted than maternally transmitted, which the authors attributed to a maternal effect, most likely Cas9 expression in the embryo leading to indels at the gRNA cleavage site (Gantz et al., 2015). Another possibility is that gene drive efficiency in mosquitoes could be modulated due to maternal or paternal imprinting. However, this is yet to be investigated for a Cas9-based HEG in mosquitoes.

A second study of a gene drive system implemented in *An. gambiae* in 2016 demonstrated in principle the use of gene drives as a vector control strategy (Hammond et al., 2016). A Cas9 homing endonuclease gene disrupted targeting female fertility genes and showed a high drive efficiency for four generations, in some cases transmitting to over 99% of offspring from one generation to the next (Hammond et al., 2016). However, following this development it became clear that the evolution of resistance against gene drives will be the greatest obstacle to overcome for field implementations. In a study which followed the gene drive for suppressing female fertility in *An. gambiae* over 25 generations, starting with caged populations consisting of 600 mosquitoes, the gene drive increased to a peak of around 80% prevalence between generations 4 and 6, and then the frequency steadily decreased in the following generations (Hammond et al., 2017). Separate novel indels disrupting the nuclease cleavage site were identified and found to be positively selected in the gene drive resistant populations, (Hammond et al., 2017) illustrating the potential of *de novo* mutations and natural selection to overcome homing endonuclease based gene drives. A follow up study demonstrated that the likelihood of resistance against a gene drive can be decreased through the selection of functionally and structurally constrained target cleavage sites (Kyrou et al. 2018). In this case, the Cas9 homing

endonuclease targeted a highly conserved region of the *Anopheles* sex differentiation gene doublesex and was able reach saturation causing total population collapse in colonies of caged *An. gambiae* (Kyrou et al., 2018). Targeting sites to avoid error-prone DNA damage repair mechanisms which can cause indels including microhomology-mediated end joining (MMEJ, also known as Alt-NHEJ) increases gene drive stability (Kyrou et al., 2018).

Ongoing research continues to demonstrate an impressive and expanding array of functionality possible with Cas9-based HEG drives. A CRISPR-based gene drive capable of simultaneous multiple gene knockout and drive has been developed in *D. melanogaster* (Kandul et al., 2020). An early concern about the use of Cas9-based gene drives had been the potential of off-target effects. Off-target effects could lead to the integration of a gene drive into multiple genomic sites, with the possibility of decreasing the affected organism's fitness and indirectly reducing the efficacy of the gene drive. However, in a study of a Cas9-based gene drive with many potential off-target sites in *An. gambiae* (as few as 2bp difference from target with an 18 bp gRNA), CRISPR induced off-target cleavage events were extremely rare and none led to gene drive (Garrood et al., 2021).

While the advances in gene drives targeting population suppression in mosquitoes have been impressive, further research has shown that the evolution of resistance against gene drives remains as a substantial hurdle to future use in wild population control. In a test of a gene drive targeting an essential gene with low genetic variance, the spread of a resistance allele eliminated the gene drive genotype within just a few generations (Fuchs et al., 2021). Population genetic models of gene drives for population suppression predict the accumulation of resistance alleles which restore fitness (Beaghton et al., 2019). Given the short generation time (10-12 days) and high reproductive potential of the malaria mosquitoes (~200 eggs per clutch, with multiple lifetime clutches per female), gene drives targeting sites with strong positive selection could have limited lifetimes in wild populations (less than 5 months assuming efficient drive is sustained for

11-12 generations). This problem could persist even if gene drive target sites are chosen for low allelic variance and intolerance of NHEJ induced indels. In some cases, the evolution of resistance against a gene drive may be delayed through the selection of functionally or structurally constrained targets, as was demonstrated with a gene drive targeting doublesex in *An. gambiae*, which maintained a high efficiency for up to 11 generations in caged populations of mosquitoes (Kyrou et al., 2019). An alternative strategy would be the development gene drives with lower fitness costs, allowing for greater population penetrance.

Many genetic intervention strategies have proposed to use gene drives to eliminate populations of disease vectors. While the development of gene drives in arthropods to decrease fertility have been the most researched strategy, this approach has the pitfall of encountering strong evolutionary resistance. Indeed, in trials on laboratory populations of *An. gambiae* in which a fertility suppressing gene drive was successfully introduced, resistance to the gene drive quickly evolved, with almost all mosquitoes containing alleles resistant to the gene drive within just 12 generations (Hammond 2017). This resistance occurred within a starting population of just 600 mosquitoes; there exists substantially more genetic variation within wild populations of mosquitoes (Miles et al., 2017). This review concludes with discussion of an alternative strategy for the implementation of gene drives, based on knowledge of olfactory driven mosquito behaviors, with reduced fitness cost.

### **Conclusions: Mosquito odorant receptor gene targets to alter vector host choice**

Anophelines which feed specifically on humans or bite humans more frequently have a much greater malaria vectorial capacity than mosquitoes which feed non-preferentially. Increased vectorial capacity can be attributed to a quadratic increase in pathogen transmission when mosquitoes bite hosts from a single species multiple times within an adult female lifespan (Dye 1992, Gary & Foster, 2001; Scott & Takken, 2012). While many genes are involved in driving

this behavior in the brain of female mosquitoes, it is worth investigating the genes which are responsible for species specific host preferences. Many species of closely related mosquitoes exhibit substantially different host preferences, such as members of the *An. gambiae* species complex. Some members of this complex (*An. gambiae* s.s. and *An. coluzzii*) exhibit a clear preference for human hosts when given a choice, while another closely related species (*An. quadriannulatus*) is more zoophilic.

ORs represent a class of chemoreceptors in *Anopheles* mosquitoes that is important for host choice, which consequently drives the transmission of diseases between permissible hosts. Several tools exist for the study of ORs, and significant groundwork has been laid in understanding host choice decisions by mosquitoes, particularly for *An. gambiae*. However, much remains to be studied. The exact ORs and host odor cues responsible for host choice are still unclear in *An. gambiae*, let alone less well studied species such as *An. stephensi*. Further, while some advances have been made, this information has yet to be translated into a functional disease prevention strategy. However, advances in molecular biology, particularly CRISPR-Cas9 gene editing, facilitate genomic modification to ORs that may be used to modify mosquito host preference.

In order to identify the ORs which are important to *Anopheles* host preferences, we selected for characterization ORs with increased transcripts in the antennae of anthropophilic, non-blood fed female mosquitoes. *Anopheles stephensi* OR homologs to *An. gambiae* ORs which met these criteria were cloned and expressed in the *Drosophila* empty neuron system. We characterized OR functions against a panel of diverse odorants designed to represent the human odor space. *Anopheles quadriannulatus* ORs which are more highly expressed in the antennae of the zoophilic species and in non-blood fed mosquitoes were similarly characterized. We identified novel odorant recognition properties of mosquito ORs for *An. quadriannulatus* Or9,

through a process of iterative selection of VOCs based upon structure-activity relationships. This data is presented in Chapter 3 of this dissertation.

Anthropophily evolved independently in the *An. stephensi* and *An. gambiae* lineages. In order to identify ORs which may be important to *An. stephensi* host seeking behavior in particular we used RNAseq to characterize the mouth part transcriptome. An OR which is highly expressed in the mouthparts, which are important to *Anopheles* host odor recognition, was characterized using electrophysiology. This odorant receptor, AsOr8, is highly responsive to many of the molecular components of human sweat odors, including 1-octen-3-ol and sulcatone. Electrophysiology was also conducted on an olfactory sensilla on the mosquito maxillary palps, known as the capitae peg. The capitae peg b neuron had a response pattern nearly identical to the response of *Drosophila* empty neurons expressing AsOr8. The only difference between the capitae peg b neuron and *Drosophila* empty neurons expressing AsOr8 is a heightened response to more diluted odorants. This data is presented in Chapter 2 of this dissertation.

Utilizing the information presented in Chapters 2 and 3 of this dissertation, we developed a proof-of-concept method to alter host choice in *An. stephensi*. We created a construct to replace expression of AsOr8 in the capitae peg b neuron with AqOr9, using Cas9 induced double-strand break followed by homologous recombination. Such a trait could be coupled to a gene drive, potentially preventing human feeding and disrupting disease transmission. We were ultimately unable to generate a transgenic mutant *An. stephensi* with Cas9 due to the challenging technical nature of the problem. Recent advances in Cas9 gene editing in mosquitoes are discussed in the concluding sections of Chapter 4, which may make implementation of gene drives to alter mosquito host choice more feasible in the near future.

## Chapter 2

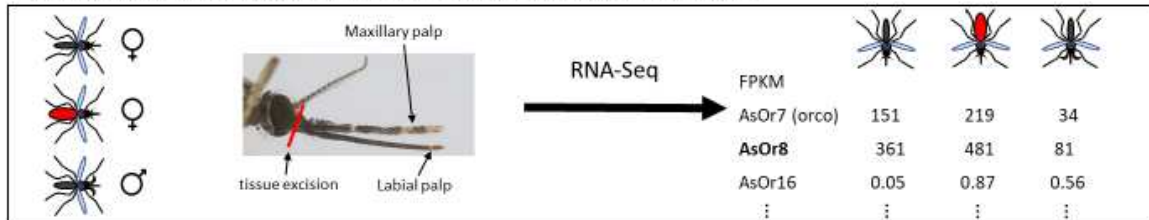
## Characterization of *Anopheles stephensi* odorant receptor 8, an abundant component of the mouthpart chemosensory transcriptome

This chapter was published in:

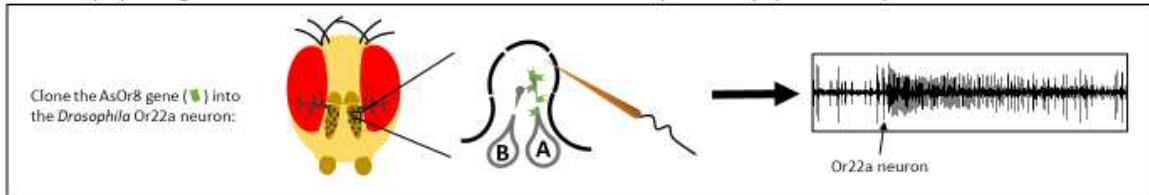
Speth, Z., Kaur, G., Mazolewski, D., Sisomphou, R., Siao, D. D. C., Pooraiouby, R., . . . Nuss, A. B. (2021). Characterization of *Anopheles stephensi* Odorant Receptor 8, an Abundant Component of the Mouthpart Chemosensory Transcriptome. *Insects*, 12(7), Article 593. <https://doi.org/10.3390/insects12070593>

Supplementary material can be accessed at: <https://doi.org/10.3390/insects12070593>

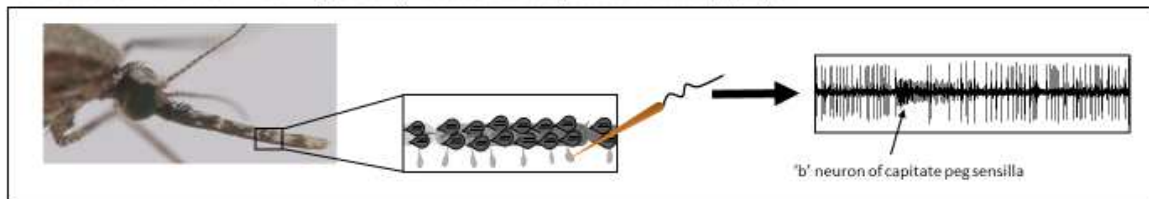
Identify chemosensory genes of interest in the *An. stephensi* mouthparts:



Electrophysiological characterization of AsOr8 within the *Drosophila* empty neuron system:



Find the neuron with matching activity on the *An. stephensi* maxillary palps:



**Abstract:** Several mosquito species within the genus *Anopheles* are vectors for human malaria, and the spread of this disease is driven by the propensity of certain species to feed preferentially on humans. The study of olfaction in mosquitoes is important to understand dynamics of host-seeking and host-selection, but the majority of these studies focus on *Anopheles gambiae* or *An. coluzzii*, both vectors of malaria in Sub-Saharan Africa. Other malaria vectors may recognize different chemical cues from potential hosts; therefore, in this study, we investigated *An. stephensi*, the south Asian malaria mosquito. We specifically focused on the mouthparts that have been much less investigated compared to antennae but are also important for host-seeking. To provide a broad view of chemoreceptor expression, RNAseq was used to examine transcriptomes from mouthparts of host-seeking females, blood-fed females, and males. Notably, AsOr8 had a high transcript abundance in all transcriptomes and was therefore cloned and expressed in the *Drosophila* empty neuron system. This permitted characterization to a panel of odorants, in part selected for their presence in the human odor profile. The responsiveness of AsOr8 to odorants was highly similar to the *An. gambiae* Or8 (AgOr8), except for sulcatone, which was detected by AsOr8 but not AgOr8. Subtle differences in receptor sensitivity to specific odorants may provide clues to species- or strain-specific approaches to host-seeking and host selection. Further exploration of the profile of *An. stephensi* chemosensory proteins may yield a better understanding of how different malaria vectors navigate host-finding and host-choice.

## 1. Introduction

Worldwide, approximately 228 million malaria cases occurred in 2019, resulting in over 400,000 deaths (WHO World malaria report 2020). In southern Asia, the predominant urban malaria vector is *Anopheles stephensi*, commonly referred to as the Asian malaria mosquito (Jiang et al., 2014). Alarmingly, *An. stephensi* was recently introduced to the Horn of Africa region, causing a spike in malaria cases and jeopardizing malaria eradication efforts there (Seyfarth et al., 2019). Its

propensity for developing in human-made habitats, including water cisterns and wells, make it uniquely suited for an urban habitat, and the anthropophilic feeding habits of the type strain of *An. stephensi* facilitate a cycle of rapid and continued human-mosquito-human malaria transmission (Sinka et al., 2011). Despite significant progress in preventing malaria cases, particularly in the last decade, gains have plateaued since 2015 suggesting limitations of current strategies in use and that additional approaches are needed (WHO World malaria report 2020). Understanding factors that govern mosquito host-seeking and host choice is one area that may lead to strategies for preventing disease transmission.

Mosquitoes possess three main classes of chemoreceptors for detecting environmental chemicals via smell or taste: odorant receptors (Ors), ionotropic receptors (Irs), and gustatory receptors (Grs). All Ors require pairing of an odorant receptor co-receptor (Orco) to one of multiple “tuning” Ors to function. In addition, odorant-binding proteins (OBPs) function to shuttle odorants through the sensillar lymph to the chemoreceptors in the neuronal membrane (McBride et al., 2016). Humans emit several odorants from breath, the skin, and the skin microbiome that are attractive to mosquitoes. These odorants include CO<sub>2</sub>, common to all respiring animals and attractive to host-seeking mosquitoes generally, but specific odorants more abundant in humans are additionally attractive to human-specialist mosquitoes. For instance, specific odorant receptors are receptive to a selection of human-emitted odorants in *Anopheles gambiae* (Carey et al., 2010; Wang et al., 2010). Additionally, several Ors are upregulated in the antennae of host-seeking female *An. gambiae* or *Anopheles coluzzii* in comparison to blood fed females, males, or the cattle-preferring *Anopheles quadriannulatus* (Pitts et al., 2011; Rinker et al., 2013a; Rinker et al., 2013b; Athrey et al., 2017). However, different mosquito species may be attracted to different components of the human-emitted odor spectrum. *Aedes aegypti*, for instance, is highly attracted to lactic acid, an abundant odorant emitted from human skin residues (Raji et al., 2019). In contrast, *An. gambiae* and *An. coluzzii* are only weakly attracted to lactic

acid, but instead respond more strongly to ammonia, which is abundant in human sweat (McBride et al., 2016). Much remains to be explored regarding chemoreception and host preference in mosquito species other than model vector species such as *An. gambiae*, *An. coluzzii*, and *Ae. aegypti*. The availability of additional Anopheline genomes continues to expand and facilitates the characterization of homologous odorant receptors in other species (Neafsey et al., 2015).

Although antennae play a strong role in mosquito chemosensation, the mouthparts (principally the maxillary palps, and the labella) also have critical host-seeking roles. For instance, removal of the maxillary palps in *An. stephensi* females significantly reduced the ability of mosquitoes to find a host, similar to the reduction in host-finding capabilities in antennae-less females (Maekawa et al., 2011). We therefore used RNAseq to explore the chemosensory repertoire of the mouthparts of *An. stephensi* to better understand odor perception in this set of sensory structures. Among other chemoreceptors and OBPs, the odorant receptor Or8 was abundantly expressed in the mouthparts. To better understand this receptor's role in host-seeking and selection, we used electrophysiology approaches to characterize this receptor's response to a panel of odorants including human volatiles. We further characterized the response of the capitite peg of *An. stephensi* maxillary palps to the odorant panel and found a similar response profile to the heterologously expressed AsOr8.

## 2. Materials and Methods

### 2.1 Mosquito rearing

*Anopheles stephensi* colonies (STE2, MRA-128 strain, obtained from MR4, origin: Delhi, India) were maintained at 28°C and 70% relative humidity on a 16:8 photoperiod (light:dark) in a dedicated insectary as previously described [15]. Briefly, first instar larvae were counted into rearing pans and fed daily on a ground fish food diet (Tetramin®, Melle, Germany) until pupation. Adults were provided with water and 10% sucrose solution ab libitum for regular

colony maintenance. For egg production, adult females were maintained with an artificial membrane feeder on bovine blood (Sharma et al., 2020).

## 2.2 *An. stephensi* Mouthpart Transcriptome

### 2.2.1 RNA sample collection and sequencing

The entire complement of mouthparts, including the maxillary palpi and proboscis, were dissected from cold-anesthetized adult mosquitoes and placed in TRIzol reagent (Invitrogen, Carlsbad, California, USA) on ice. 500 mouthparts were collected per sample. For females, one group was blood fed 12 h prior to mouthpart collection, while the other female group and males were maintained on 10% sucrose only, as per normal colony conditions. Due to limited resources, only single replicate collections were made for each condition. All mosquitoes were 5 days post-emergence, and all collections were performed at dusk, approximately 1-2 h before scotophase.

Total RNA was extracted from mouthparts by grinding with plastic pestles and using the Direct-zol RNA Miniprep Kit (Zymo Research, Irvine, California, USA) and treated with DNase I (Ambion, Austin, Texas, USA) with incubation for 30 min at 37°C, according to manufacturer's protocols. RNA integrity for each sample was determined using an Agilent 2100 Bioanalyzer. PolyA tail enriched RNA libraries were prepared by the Nevada Genomics Center using an Illumina TruSeq stranded mRNA Prep kit (Illumina Inc., Hayward CA, USA). Paired end sequencing was performed on an Illumina NextSeq 550 using a High Output Kit, Version 2, 150 cycles, flow cell (Illumina Inc., San Diego, California, USA).

### 2.2.2 Quality control of raw reads

Sequence read pairs were filtered and trimmed to remove low-quality reads, adapters, and artifacts using Trimmomatic software v0.36 with default parameters (Bolger et al., 2014). Quality control (QC) of reads were evaluated on each individual sample using FastQC v0.11.9 (Andrews et al., 2010; <http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) prior to and after

trimming. In addition, the FastQC reports were unified into a master report for all samples pre- and post-trimming using the MultiQC software v1.9 (Ewels et al., 2016) and the RSeQC package v4.0. After initial trimming and QC, the STAR aligner v2.7.5c (Dobin et al., 2013) was used to align reads to the *An. stephensi* SDA-500 genome assembly. The *An. stephensi* SDA-500 reference (GCA\_000349045.1 - VEuPathDB release # / date: release 49 / 05-NOV-20) and corresponding genome annotation (AsteS1.8) was downloaded from vectorbase.org. Insert sizes were calculated via PicardTools CollectInsertSizeMetrics v2.24.0 (<http://broadinstitute.github.io/picard/>). Following alignment, featureCounts v2.0.0 software from the subread package (Liao et al., 2014) produced total counts, which were used to calculate TPM (transcripts per million) values (Li et al., 2011; Wagner et al., 2012) and FPKM (fragments per kilobase of transcript per million fragments mapped) values with the R package countToFPKM v2.0.0 (DOI: 10.1093/bioinformatics/btt656). Annotation was retrieved from VectorBase on 01/22/2021.

### 2.3 Electrophysiological *AsOR8* characterization in the *Drosophila empty neuron* system

#### 2.3.1 Cloning

*AsOr8* sequences were identified by using the tBLASTn function in Vectorbase (Giraldo-Calderon et al., 2015) using the *An. gambiae* Or8 sequence as a query (Pitts et al., 2011; Lu et al., 2007). Sequences annotated as Or8 were returned from the Indian strain annotation (ASTEI08712-RA) and SDA-500 annotation (ASTE009819-RA) encoding predicted 409 or 401 amino acid products, respectively. Regardless, 5' and 3' ends of the open reading frame were identical between the two sequences and primers were designed accordingly. Primers were designed with restriction site overhangs (Forward: *AsOr8F1*EcoRI [ATAGAATTCACCATGCCACCAGCAAACCTCTACC, T<sub>m</sub> = 57.6 °C]; Reverse: *AsOr8R1*XbaI [CGCTCTAGATTACTTCACATTCTTCTCATTGGGTTTCG, T<sub>m</sub> = 57.1 °C]) for

cloning. RNA was extracted as above, from 50 heads of 5-day old *An. stephensi* females maintained on 10% sucrose, collected at 1 h prior to scotophase. cDNA was prepared using reverse transcription with the Superscript IV kit (Invitrogen, Carlsbad, California, USA). AsOr8 transcripts were amplified with Phusion DNA polymerase (Phusion Green HSII, Thermo Scientific) using a 2-step PCR protocol: 98°C for 15 s, followed by annealing at 65-56°C (-1°C each cycle) for 30 s and extension at 72°C for 75 s for 10 cycles, then followed by 98°C for 15 s, annealing at 63°C for 30 s and extension at 72°C for 1 min 15 s for 30 cycles.

PCR products were separated by gel electrophoresis, excised, and purified using the Zymoclean Gel-DNA Recovery Kit (Zymo Research, Irvine, California, USA). Products were digested with EcoRI and XbaI in Cutsmart buffer (New England Biolabs, Ipswich, Massachusetts, USA) at 37°C for 2 h, followed by heat inactivation of the restriction enzymes by incubating at 65°C for 20 min. The pUAST vector was prepared for ligation following the same restriction digestion protocol [26]. For the ligation reaction, 20 fmol of digested pUAST was mixed with 80 fmol of digested insert PCR product and incubated with T4 DNA ligase (New England Biolabs) at room temperature for 1 h, followed by inactivation of the ligase by incubation at 65°C for 10 min. The ligation product was then used to heat shock transform Top10 cells (Invitrogen, Carlsbad, California, USA) following the manufacturer's protocol.

Cells were plated and colonies were checked with PCR for the AsOr8 insert. Cells containing the AsOr8 insert were grown and plasmid was extracted using the Qiagen Plasmid Midi Kit (Qiagen, Germantown, MD, USA). Plasmids were Sanger sequenced (Genewiz, South Plainfield, NJ, USA) to confirm the AsOr8 sequence was cloned accurately and without introns or frameshifts.

### 2.3.2 *Drosophila melanogaster* stocks and transgenes

AsOr8 was expressed in the *Drosophila melanogaster* empty neuron system for electrophysiological characterization (Dobritsa et al., 2003; Hallem et al., 2004). *Drosophila melanogaster* embryos were transformed by injecting pUAST containing the AsOr8 insert (Rainbow Transgenic Flies, Inc., Camarilla, CA, USA). Transformants were crossed with a w<sup>1118</sup>;cyo/sco;Tm6/mkrs balancer line and screened for UAS-AsOr8 insertion on the third chromosome. These flies were crossed with the  $\Delta$ halo line to generate a  $\Delta$ halo/cyo;UAS-AsOr8/mkrs line ((wt 1118);  $\Delta$ halo/cyo; 22a-Gal4/mkrs flies were kindly provided by Dr. Anandasankar Ray, University of California, Riverside, CA). The flies which were characterized by electrophysiology were obtained by crossing the w<sup>1118</sup>;  $\Delta$ halo/cyo;UAS-AsOr8/mkrs flies with w<sup>1118</sup>;  $\Delta$ halo/cyo; 22a-Gal4/mkrs flies and selecting for the w<sup>1118</sup>;  $\Delta$ halo/ $\Delta$ halo ;UAS-AsOr8/Or22a-Gal4 flies.

### 2.3.3 Electrophysiology

Single unit extracellular recordings in the *Drosophila* empty neuron were performed on 4-14 day old  $\Delta$ halo/ $\Delta$ halo; UAS-AsOr8/22a-Gal4 flies and adapted from methods described before (Dobritsa et al., 2003; Mathew et al., 2013). For all recordings, the ground electrode, a pulled glass capillary pipette filled with Ephrussi and Beadle solution (Ashburner, 1989), was slipped over an AgCl coated silver wire and placed into the flies' right eye. For the recording electrode, a tungsten microfilament was placed into direct contact with an Ab3 sensilla. The signal was passed through an Iso-DAM8a impedance amplifier (WPI), with the high-pass filter set to 100 Hz and the low-pass filter set to 3 kHz, and digitized at 10667 Hz using a Syntech IDAC4 Intelligent Data Acquisition Controller.

Odorants were diluted 1:100 v/v in paraffin oil and were mobilized with clean, humidified air directed towards the fly antenna at 1.4 L/min (37.5 mL/s). During stimulus presentation, a 0.5 s pulse of 3.75 mL/s amplitude was directed through the shaft of a Pasteur

pipette containing 50  $\mu$ L of 1:100 diluted (vol./vol.) odorant placed on a 13 mm Whatman filter paper placed inside the Pasteur pipette. Controlled odor pulses were delivered through the odor cartridges using a Syntech stimulus controller (CS-55). Odor cartridges were used no more than three times prior to replacement with a new cartridge.

Odorants were presented to flies in sets of up to 10 odorants, including solvent control and paraffin oil (PO), while recording from a single fly sensilla. The odorant presentation order was randomized between recordings from single sensilla. Up to three sensilla per fly were used for recordings. Responses were stored and characterized using Autospike (Syntech). Action potentials were counted manually over the 500 ms period when the odor pulse passed the antenna. The average solvent response to paraffin oil was subtracted from the reported action potential firing rates.

For the mosquito capitata peg recordings, female *An. stephensi*, 3-5 days post-emergence, were fixed in a similar manner to *D. melanogaster*, with the ground electrode placed into the right eye of the mosquito. The maxillary palp was affixed onto a piece of double-sided tape, ventral side up, and the recording electrode was placed into contact with a capitata peg on the third or fourth palp segment. Each response to an individual odor was recorded on a different capitata peg sensilla.

#### 2.4 Odorant panel selection:

Odorants were selected to represent human and animal odors across a broad range of chemical classes, as well as heterocyclics and aromatics representative of ecologically relevant plant phytochemicals which elicit responses from a large fraction of characterized mosquito odorant receptors (Table S2) (Carey et al., 2010; Wang et al., 2010). Human and animal odors consisted primarily of alcohols, aldehydes, and ketones. Carboxylic acids and amines were underrepresented in this panel, despite being potent mosquito attractants. These are not

commonly perceived by dipteran Ors, although Irs respond robustly to these odorants (Pitts et al., 2017).

### 3. Results

#### 3.1 *An. stephensi* mouthpart transcriptome reveals high expression of *Or8*

Overall, the mapping rate (~82.5%) and mapped proper pairs (~95.1%) of *An. stephensi* mouthpart transcripts indicated successful mapping. Furthermore, the Proper Pairs metric (~95%) confirmed these results indicating that forward and reverse strand were properly mapped, as opposed to reverse/reverse or forward/forward mapped reads (improper pairs) which were excluded from expression quantification. STAR alignment resulted in 13,611 mapped transcripts, of which 3,056 showed no expression in any of the samples. Overall alignment rate was between 81.1-83.7% (Table 1). See Table S1 for a merged file including: VectorBase annotation, TPMs, FPKMs, and feature counts.

Outside of genome-wide annotations (Jiang et al., 2014), the full complement of Ors, Irs, Grs, and OBPs in *An. stephensi* has not been extensively characterized. In comparison to the more highly characterized *An. gambiae*, multiple chemoreceptor and OBP homologs were not found in *An. stephensi*, or were not annotated as such (Table 2). Several *An. stephensi* genes were annotated as unnumbered Ors, Irs, Grs, or OBPs, or as uncharacterized proteins, but tBLASTn searches with *An. gambiae* homologs in Vectorbase revealed multiple apparent *An. stephensi* homologs (see Table S1 for full annotation list). Ors, Irs, and OBPs all had less annotated genes in *An. stephensi* than *An. gambiae*, and several in each group were incompletely annotated or unannotated. Whether the reduction of genes in *An. stephensi* is a result of incomplete genome assembly or whether gene loss or duplication occurred is unclear. All Grs in *An. gambiae* were present in *An. stephensi*, although several were also incompletely annotated or unannotated.

The *An. stephensi* mouthpart RNAseq revealed that transcripts were detected for approximately half of the identified *An. stephensi* Ir, Gr, and OBP genes, and approximately a third of Or genes (Table S1). However, a low threshold cutoff was applied at 1% of the highest TPM within each of the chemoreceptor groups or the OBPs which resulted in approximately 7-19 transcripts detected in each group (Figure 1). The predominant Ors with high transcript abundance in *An. stephensi* mouthparts were Or7 (Orco), Or8, and Or28 (Figure 1A). Ir co-receptors Ir25a and Ir76b were also detected in the mouthparts, yet transcripts for the co-receptor Ir8a were negligible. 17 additional Ir transcripts were detected above the 1% cutoff (Figure 1B). Gr22, Gr23, and Gr24, presumptive *An. stephensi* CO<sub>2</sub> receptor orthologs (Lu et al., 2007), were highly expressed in the mouthparts. Gr31 and Gr52 were also consistently expressed, at lower abundance, in addition to trace detection of other Grs (Figure 1C). The most abundant odorant-binding proteins expressed were OBP13, OBP26, OBP48, and OBP57, and to a lesser degree, OBP9, OBP10, and OBP54 (Figure 1D). Transcript abundance between sexes or by feeding status was proportionally similar, with less expression overall detected in males. However, the collection of single replicates for sex/feeding status prevented statistical comparison of expression levels.

### 3.2 *AsOr8* is receptive to alcohols and ketones in the human volatile spectrum

To further understand the role of the highly expressed *AsOr8* in the mouthparts, we explored the sensitivity of this receptor to assorted odorants by expressing it in the *Drosophila* empty neuron system. This is an *in vivo* expression system in the fly antenna, in which individual Ors (from *D. melanogaster* or a mosquito species) can be expressed in a mutant neuron that lacks an endogenous functional Or (Carey et al., 2010; Dobritsa et al., 2003). The cloned *AsOr8* sequence encoded a 401 amino acid protein nearly identical to the *An. stephensi* SDA-500 annotation, but with substitutions at positions 241 (Asp to Gly) and 248 (Ala to Glu) (GenBank#

MW076538). The cloned sequence is 89.5% identical to *An. gambiae* Or8 (AgOr8) (Lu et al., 2007; Accession: AGAP001912) (Figure 2). Extracellular loop regions were identical between AsOr8 and AgOr8 except for four amino acid differences in extracellular loop 2 (Figure 2).

As expressed in the *Drosophila* empty neuron system, AsOr8 was sensitive to 6-8 carbon alcohols and ketones, with 1-hepten-3-ol eliciting the greatest activity at high concentrations. 3-octenol, 1-hexanol, sulcatone (6-methyl-5-hepten-2-one), 1-octen-3-ol, and 2-heptanone also elicited strong responses (Figs. 3, 4A). At lower odorant concentrations ( $10^{-4}$ ,  $10^{-6}$  vol./vol. dilutions), AsOr8 exhibited the greatest sensitivity to 1-octen-3-ol, and was also sensitive to 3-octanone and 1-hepten-3-ol, but the response to sulcatone was insignificant (Figure 4B, C).

### 3.3 Maxillary Palp Capitata Peg recordings mirror AsOr8 recordings

To explore odor responsiveness of the mouthparts *in vivo*, single sensilla electrophysiological recordings from female *An. stephensi* capitata pegs located on the maxillary palps were performed. Although the specific expression distribution of AsOr8 in capitata pegs was unavailable, the capitata peg 'B' (cpB) neuron responses mirrored those taken from the empty neuron in *D. melanogaster* exogenously expressing AsOr8 (Figure 4B, C). The cpB neuron responded strongly to 1-hepten-3-ol, sulcatone, 1-octen-3-ol, and 3-octanone at high concentrations ( $10^{-2}$ ) (Figure 4A), while exhibiting the greatest sensitivity towards 1-octen-3-ol. For each odorant tested at lower concentrations, the cpB neuron exhibited a greater response than the *Drosophila* empty neuron expressing AsOr8, with the exception of 1-octen-3-ol (Figure 4B, C).

## 4. Discussion

The proboscis and maxillary palps play a crucial role in host-seeking behavior of female mosquitoes, as has been demonstrated in *An. stephensi* (Maekawa et al., 2011). Here we report on the first *An. stephensi* mouthpart transcriptome to more fully explore chemosensation of this

important sensory appendage. Notably, transcripts for AsOr8 were highly abundant and we further characterized this receptor to better understand its potential chemosensory role, particularly with regard to host-seeking and volatiles found in the human odor spectrum. In mosquitoes, Or8 has a conserved role in detecting (R)-1-octen-3-ol (Carey et al., 2010; Lu et al., 2007; Bohbot et al., 2009; Grant et al., 2011; Dekel et al., 2016). In blood-feeding species, Or8 is also activated by several compounds emitted by vertebrates, such as 1-hepten-3-ol, sulcatone, and 2-heptanone (Carey et al., 2010; Wang et al., 2010; Lu et al., 2007). Curiously, the ability to detect (R)-1-octen-3-ol is retained in *Toxorhynchites ambionensis* Or8, but in this species, which does not seek blood meals, the receptor was much more narrowly tuned. It was not responsive to 1-hepten-3-ol, 2-heptanone, or 3-octanone, and it is suspected to function in detecting plant volatiles (Dekel et al., 2016).

In the current study, AsOr8 responded to similar vertebrate-emitted odorants to the homolog of its closest characterized relative, AgOr8 with the exception of sulcatone which activated AsOr8 in our study, but did not elicit a strong response from AgOr8 (Carey et al., 2010; Wang et al., 2010; Lu et al., 2007). Functionally, the activation of AsOr8 by sulcatone (Figure 3), and the converse unresponsiveness of AgOr8 to this compound, is interesting in light of this compound's role in human host selection in *Ae. aegypti* (McBride et al., 2014). These receptors share high amino acid conservation, but specific amino acid changes in Or8 may be responsible for the difference in response of AsOr8 and AgOr8 to sulcatone (Figure 2). Although the key amino acids for odorant selectivity of Or8 have not been characterized, a mutation screen of AgOrs 13 and 15 suggested that changes to amino acids in extracellular loops 2 and 3, and transmembrane regions V-VII shifted odorant specificity in these receptors (Hughes et al., 2014). These regions in AsOr8 and AgOr8 were identical except for four amino acid changes in extracellular loop 2 (Figure 2) and are possible candidates for a structural basis for odor sensitivity shift between these species' receptors. However, the b neuron of the *An. gambiae*

capitate peg is responsive to sulcatone, to a slightly lesser degree, than the b neuron of *An. stephensi* (Lu et al., 2007). This raises the possibility that structural differences between these receptors may be compensated for by other components of the OSN environment, such as OBPs. In addition, several AgOrs do display some sensitivity to sulcatone, including AgOr30, AgOr39, AgOr57, AgOr75, and the maxillary palp-expressed AgOr28 (Carey et al., 2010; Wang et al., 2010; Omondi et al., 2019). Further characterization of *An. stephensi* may demonstrate redundancy of sulcatone detection in other Ors also.

The cpB neuron and the fly empty neuron expressing AsOr8 were both most responsive to 1-octen-3-ol, 1-hepten-3-ol and 3-octanone at the lowest odorant concentrations tested (Figure 4B and 4C). In the natural environment, insects encounter individual odorants at low concentrations within complex mixtures. Detection of these odorants and structurally similar molecules is therefore likely to be the most biologically relevant function of the cpB neuron and AsOr8. Importantly, both 1-hepten-3-ol and 1-octen-3-ol are higher abundance components of human odors (Bernier et al., 2000). Comparison of AsOr8 responses expressed in the *Drosophila* empty neuron system to the cpB neuron of *An. stephensi* maxillary palpi showed similar responses to odorants, yet with increased firing rate in the cpB neuron readings. The increase in apparent sensitivity of the cpB neuron to some odorants could be attributed to differences in the interaction of AsOr8 with Orco of *D. melanogaster* and *An. stephensi*, but could also be influenced by other differences between the neuronal environments, including differences between secreted soluble proteins such as OBPs. On the maxillary palps of Anopheles mosquitoes there is only one sensilla type, the capitate pegs. On the *An. gambiae* maxillary palps, the capitate peg sensilla houses the CO<sub>2</sub> detecting neuron which expresses Gr22, Gr23, and Gr24 paired with two odor sensory neurons expressing Or28 and Or48, respectively (Lu et al., 2007). The RNA-seq and electrophysiology results presented in this study support a conserved neuronal arrangement in the *An. stephensi* capitate pegs.

In addition to our characterization of AsOr8, comparing *An. stephensi* mouthpart transcript abundance of chemosensory receptors and OBPs to previously published *An. coluzzii* and *An. quadriannulatus* maxillary palpi transcriptomes (Pitts et al., 2011; Athrey et al., 2017) showed broad similarity with some exceptions. The high expression of Orco, Or8, and Or28, Ir25a and Ir76b, and Gr22-24 homologs in *An. stephensi* is consistent with the maxillary palpi expression of other anophelines [8, 11, 25, 40]. Ir7s, Ir31a, Ir93a, and Ir135 notably had greater transcript abundance by proportion in *An. stephensi* than in other anophelines. Conversely, Ir75k is more highly expressed in *An. coluzzii* and *An. quadriannulatus*. Ir100a is prominently expressed in both *An. coluzzii* and *An. quadriannulatus*, but *An. stephensi* apparently lacks this homolog. Gr52 is expressed in the mouthparts of all species except for *An. quadriannulatus* and has proportionately high expression in males and females. However, Gr31 was expressed in *An. stephensi*, but was not detected in the maxillary palpi of other anophelines. OBP expression follows a conserved pattern in anophelines, with high expression of OBP 48 and 57, and minor expression of OBP10 and 25. A direct homolog for AgOBP25 was not found in *An. stephensi*, but instead, detectable expression of AsOBP26 may suggest a functional, if divergent, homolog. OBP13 expression was notably higher in *An. stephensi* compared to other anophelines. It is interesting to speculate on differing expression patterns between *An. stephensi* and Anophelines from prior studies as it relates to species-specific odor perception and attraction. However, some caution is warranted. These differences may be attributable to differences in study conditions, such as age and time of day, and the precise tissues examined (maxillary palps versus all structures within the mouthparts). For instance, expression in the labellum of *An. coluzzii* varied markedly from expression in the maxillary palpi of this species (Saveer et al., 2018). This may explain the apparent differential expression of OBPs 13 and 54 in *An. stephensi* which are not detectably expressed in *An. coluzzii* or *An. quadriannulatus* maxillary palpi transcriptomes, but OBPs 14 and 54 are expressed in the labium of *An. coluzzii* (Saveer et al.,

2018). In addition, *An. stephensi* apparently lacks homologs described in the archetypical *An. gambiae*, although whether this is a result of gene loss in *An. stephensi*, gene duplication in the *An. gambiae* lineage, or incomplete *An. stephensi* genome assembly remains to be determined. A recent genome reassembly of *An. stephensi* reported 54 Ors (Chida et al., 2020), in contrast to the 59 apparent Ors we identified in the current study. A thorough re-annotation of chemoreceptors, and the OBPs particularly, may be warranted. Further study will be required to elucidate whether the transcriptome generated in the current study accurately reflects consistent expression differences between species or under different physiological states.

## 5. Conclusions

Overall, our study provides an initial exploration into the expressed chemosensory repertoire of the mouthparts of adult *An. stephensi*, an important malaria vector in South Asia. AsOr8 is a highly expressed component in the mouthparts and is sensitive to 1-octen-3-ol, as well as several human-emitted odorants. Interestingly, characterization of this receptor suggested differences in detection of sulcatone, a component of the human volatile spectrum, compared to a homolog in the well-studied model malaria vector, *An. gambiae*, and could lay the foundation for further characterizing structure-function relationships of mosquito odor receptors. Further exploration of chemosensory protein expression in *An. stephensi*, particularly in the antennae, may highlight differences that elucidate species- or strain-specific dynamics of host-seeking, and, importantly, human host choice, a behavior that strongly impacts human disease transmission.

**Supplementary Materials:** The following are available online at [www.mdpi.com/xxx/s1](http://www.mdpi.com/xxx/s1):

**Table S1:** RNAseq data for chemoreceptors and OBPs: TPMs and feature counts of *An. stephensi* Ors, Irs, Grs, and OBPs. Vectorbase Gene IDs are listed for all *An. stephensi* genes. Red text in

the Gene Name column indicates that no *An. stephensi* ortholog to an *An. gambiae* gene was found.

**Table S2:** AsOr8 response to odorant panel: Spikes/sec ( $\pm$ SD) of all selected odorants tested against AsOr8 as expressed in the *Drosophila* empty neuron system. Odorants that have a human or animal association are noted.

**Author Contributions:** Conceptualization: ABN, MG-N, DM; formal analysis: ZS, RP, ABN, GK, HV-G, JP; investigation: ZS, DM, RS, DS; data curation: ABN, GK, HV-G, JP; writing-original draft preparation: ZS, ABN, DM; Writing-review and editing: ZS, ABN, MG-N, DM; project administration: ABN, DM; funding acquisition: ABN, DM. All authors have read and agreed to the published version of the manuscript.

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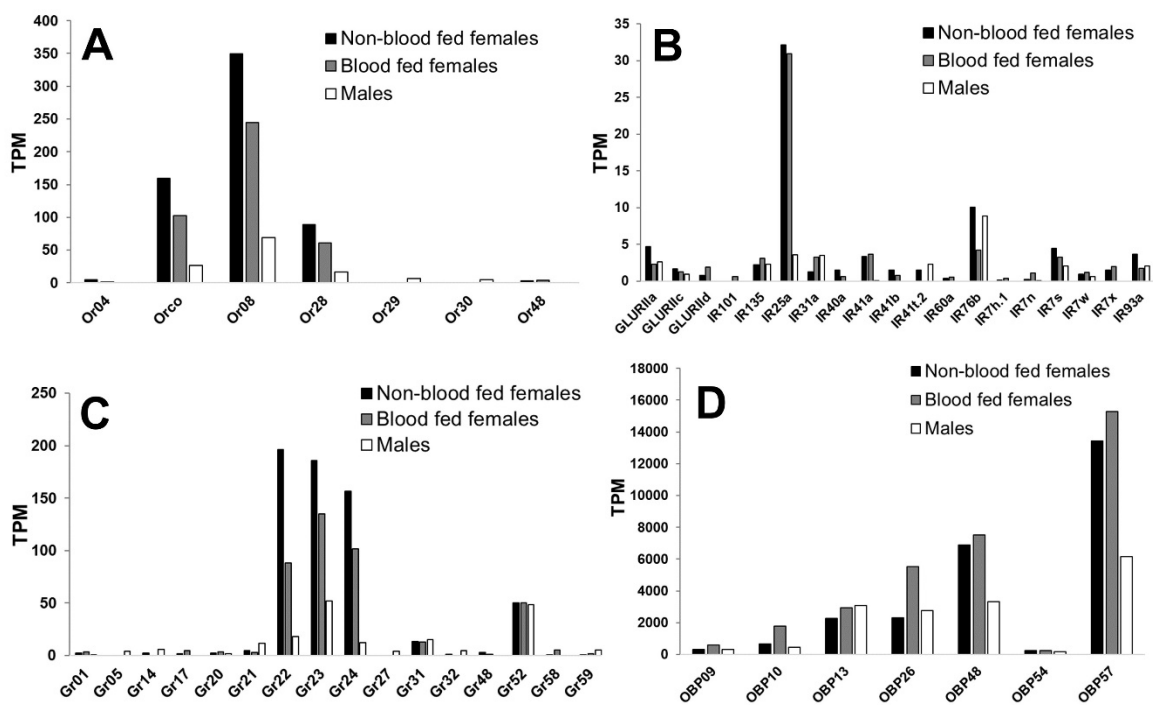
**Conflicts of Interest:** The authors declare no conflicts of interest.

**Table 1: RNAseq mapping quality to the *An. stephensi* SDA-500 genome assembly.**

Sample	Total read pairs	Mapped reads	Overall alignment rate	Proper Pairs	Fragment Length	Total Feature Counts	Total FPKMs
Blood-fed female	54,257,482	45,434,941	83.7%	94.9%	171	29,106,087	1,493,597
Non-fed female	47,808,166	38,786,401	81.1%	95.1%	179	25,379,805	1,382,585
Male	36,364,670	30,038,819	82.6%	95.2%	152	20,034,497	1,279,250

**Table 2: *An. stephensi* Or, Ir, Gr, and OBP genes corresponding to annotated *An. gambiae* homologs.**

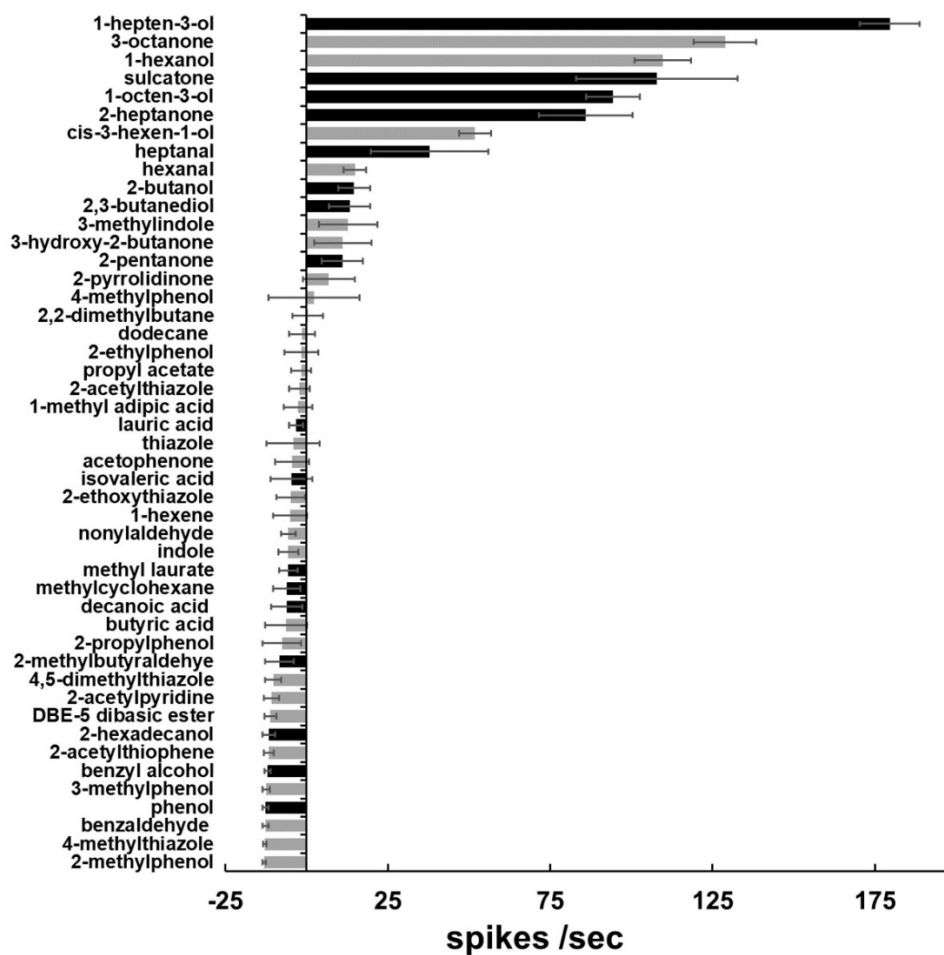
	Or	Ir	Gr	OBP
Total <i>An. stephensi</i> chemosensory genes (number less than <i>An. gambiae</i> )	59 (17)	37 (12)	60 (0)	42 (24)
Unnumbered or unannotated	17	14	8	35
Expression detected	18	22	35	30



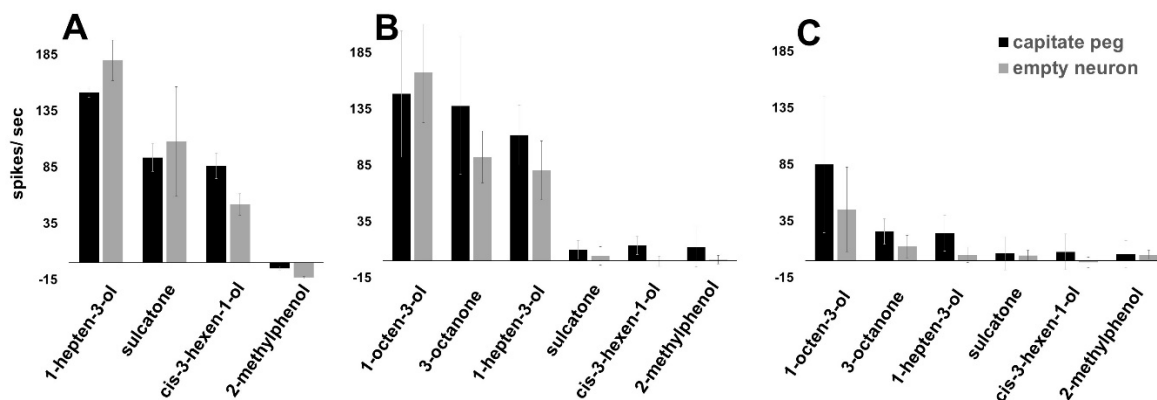
**Figure 1: Transcript abundance of chemosensory genes.** Transcript abundance of (A) odorant receptors, (B) ionotropic receptors, (C) gustatory receptors, and (D) odorant-binding proteins in the mouthparts of *An. stephensi* non-blood fed females (black bars), 12 h blood fed females (gray bars), and males (white bars).

		I _____	
AsOr8	MPPANS	TDLVQFESFIRVPEIFFTMIQVARYGEPRTLQAHLKQLLEFWSSCANTGFCLL	60
AgOr8	--MDPPT	DELVRVFESFIRVPEIFFFAMIGVARYGEPKRTLRAVLRKHLLEFWSSCINTGFCLV	58
		II _____	
AsOr8	IEHIYFVKAAGNFTNFLQLTALAPCMGFTALSFVKIMTIQLNGTKLTDMLHRLDALFPKS	120	
AgOr8	IEHIYFVKAAGNFTNFLQLTALAPCMGFTALSFVKIMTIKLNETKLTDLMLHRLDALFPST	118	
		III _____ **	
AsOr8	AALQERYGVFQYNRESFVVMKSFSILYMTLIWMFNLLPLVSMVAGYCADGTWHKQLPYFM	180	
AgOr8	VALQERYGVFQYNRESFVVMKSFSILYMTLIWMFNLLPLVSMVAGYVSDGTWHKQLPYFM	178	
		* *IV _____	
AsOr8	WYWDWHEPGYFVTFVLHQNWGGFVSAVFYLSLTDLMFCAIVLLVCLQFDIVAYRLKHAEP	240	
AgOr8	WYWDWHRPGYFAVTFVLHQNWGGFVSAVFYLSLTDLMFCAIVLLVCLQFDIVAYRLSHAEP	238	
		+ + V _____ VI _____	
AsOr8	GDQQLHECVRIHQAVIELCSELEHMFSPSLLVNFLLSSSVIICLVGFQATAGITPADLFK	300	
AgOr8	DDHQELVGCVRIHQAVIELCNELEHMFSPSLLVNFLLSSSVIICLVGFQATAGITPADLFK	298	
		_____	
AsOr8	FVLFLVSSLVQVFLLCYYGNKLIIVASSQIPYSAFEGOWIGASVYQSRLLFVMLRSTTVQ	360	
AgOr8	FVLFLVSSLVQVFLLCYYGNKLIIVASSQIPYSAFEGNWIGASVSYQSRLLFVMLRSTTVQ	358	
		VII _____	
AsOr8	KLTKLKFSLVSLASYSKILSTSFYSYFTLLKALYEPNEKNVK	401	
AgOr8	KLTKLKFSLVSLASYSKILSTSFYSYFTLLKAMYEPNEKMK	399	

**Figure 2: Primary structure comparison of AsOR8 and AgOR8.** A: Amino acid alignment of AsOR8 and AgOR8. Highlighted areas indicate identical and conserved residues as designated by ClustalOmega [32]. Black = identical residues; dark gray = strongly similar residues; light gray = weakly similar residues (for amino acid similarity groups, see: [http://www.clustal.org/download/clustalx\\_help.html](http://www.clustal.org/download/clustalx_help.html)). Predicted transmembrane (TM) domains I-VII are indicated as a line above the alignment (N-terminus is cytosolic). "+" above the sequence indicates an amino acid change in the sequence determined in this study from the predicted *An. stephensi* SDA-500 genome sequence. Differences in extracellular loop 2 between species are noted with "\*\*".



**Figure 3: Responses of the *Drosophila* empty neuron expressing AsOr8 to selected volatiles.** (A) Response of AsOr8 to selected volatiles, diluted 1:100 (vol./vol.) in paraffin oil, in the *Drosophila* empty neuron system. Black bars: odorant detected in human emanations. Gray bars: odorant not detected in human emanations (see Table S2 for responses to all compounds tested).



**Figure 4: The *An. stephensi* cpB neuron responds to the same odorants as the *Drosophila* empty neuron expressing AsOr8.** Responses of the *An. stephensi* maxillary palp cpB neuron (black bars) or the *Drosophila* empty neuron expressing AsOr8 (gray bars) to odorants which elicit the strongest responses, at  $10^{-2}$  (A),  $10^{-4}$  (B), and  $10^{-6}$  (C) (vol./vol.) dilutions in paraffin oil. Recordings were taken from unique capitate peg sensilla on female *An. stephensi* maxillary palps or AB3 sensilla on *Drosophila* antenna. CpB counts were taken within the 500 ms window as the odor pulse passed over the *An. stephensi* maxillary palps or the antennae. At  $10^{-2}$ , the cpB neuron was activated strongly by 1-octen-3-ol and 3-octanone, but the initial spike amplitude became indistinguishable from background noise within the counting interval.

**Chapter 3:****Characterization of mosquito odorant receptors from anthropophilic and zoophilic species**

**Abstract:** Olfaction plays an important role in mosquito behaviors, including foraging for nectar and identifying potential hosts for a blood meal. Mosquitoes detect semiochemicals through odor receptors, each of which interact with specific odorants. Different mosquito species exhibit particular host preferences which contribute to the capacity to vector diseases. Odor receptors from two species of malaria competent mosquito, one anthropophilic and the other zoophilic, were characterized in a heterologously expressing olfactory receptor neuron using a panel of structurally diverse odorants. Two mosquito odor receptors, AsOr45 and AsOr76, exhibited significant inhibitory responses to odorants. A third odor receptor, AqOr9, was particularly responsive to aromatic and heterocyclic molecules. The odor receptor characterizations allowed for a cross species comparison of odor receptor function. A pattern of widespread functional conservation of ORs between *Anopheles* species which diverged up to 50 million years ago emerged, even for ORs with relatively low structural conservation.

AqOr9 response was characterized in further detail against a second odorant panel with aromatic and heterocyclic structure arising from different environmental sources, including human and animal odors, floral and plant oil scents, yeast induced fermentation, and woodsmoke. It was found that AqOr9 was highly sensitive to guaiacols, an activity not previously described for a mosquito chemosensory receptor. Guaiacols are found in various natural sources, including woodsmoke, and 4-ethylguaiacol is produced by fermenting yeast. Several of the volatile organic molecules that activated the receptor at sub-nanogram quantities are products of lignin pyrolysis. This information suggests that Or9 could be important for *Anopheles* recognition of smoke sources in the environment. The strategy applied in this study of selecting odorants with similar molecular characteristics to activating odorants identified from a screen of structurally diverse volatile organic compounds could be applied to uncover previously hidden chemosensory functions of odorant receptors.

## 1. Introduction:

Malaria is a devastating disease spread by *Anopheles* mosquitoes, killing over 400,000 people every year (WHO world malaria report 2020). While many *Anopheles spp.* are vector competent for malaria, only a few species are responsible for the vast majority of human cases, including *Anopheles stephensi*, the predominant urban vector of human malaria in India and the Middle-East (Jiang et al., 2014). The few species which do vector human malaria exhibit a preference for feeding on human hosts (Takken & Verhulst, 2013). One determinant of mosquito host choice is attraction to odors. Among mosquito chemosensory gene families, odor receptors (ORs) are receptive to volatile components of sweat, breath, and skin microbiota (Hallem et al.; 2004, Fox et al, 2004; Carey et al., 2010 ; Wang et al., 2010; McBride et al., 2014).

Closely related mosquito species, or even strains within a species, can exhibit different host preferences. For instance, differences in host preferences are exhibited within closely related species of the *An. gambiae* species complex, in which *An. gambiae* and *An. coluzzii* feed primarily on human hosts and are significant vectors of human malaria, while other species such as *An. quadriannulatus* and *An. amharicus* feed primarily on other animals. *An. gambiae* strongly prefers human over cattle odors in choice tests (Pates et al., 2001). In contrast, *An. quadriannulatus* exhibit a much greater attraction to cattle odors and higher concentrations of CO<sub>2</sub> than anthropophilic sibling species (Dekker & Takken 2002). Therefore genetic differences in chemosensory genes could play an important role in mosquito species' host preferences. Functional genomics has been employed to explore OR antennal expression in species with different host preference, in different physiological states, and by sex (Pitts et al., 2011; Rinker et al. 2013a; Rinker et al., 2013b; Athrey et al., 2020; Athrey et al., 2021). A handful of ORs have been suggested as associated with anthropophily or zoophily, but direct associations between feeding choice and OR expression remain to be demonstrated, except in *Aedes aegypti* (McBride et al., 2014).

Many *An. gambiae* ORs have previously been characterized in both the *Drosophila* empty neuron system and *Xenopus* oocytes (Carey et al., 2010; Wang et al., 2010). Several *An. gambiae* ORs interact with odorants emitted by humans and other animals (Carey et al., 2010; Wang et al., 2010), and could be important in host seeking behavior. However, ORs have among the highest rates of non-synonymous to synonymous single nucleotide polymorphisms of any Anopheles gene family, which is correlated with a high rate of positive selection (Neafsey et al., 2015). It therefore remains unclear whether more distantly related species, such as *An. stephensi*, would have a high degree of functional conservation of OR function with *An. gambiae* counterparts, given the approximately 50 million years of evolutionary divergence separating the two species.

Insect ORs are odorant-gated ion channels (Butterwick et al., 2018; del Marmol et al., 2021). For most insects, the odorant receptor co-receptor (orco) forms a functional heterotetrameric, odorant-gated ion channel when paired with ORs which exhibit particular odorant induced activity (Joseph & Carlson, 2015; Butterwick et al., 2018). Olfactory receptor neurons (ORNs) have both odorant-induced and spontaneous activities which are conferred primarily by the endogenous OR (Hallem et al., 2004). Many ORs confer a large increase in ORN action potential frequency of up to 200-250 action potentials per second, upon odorant exposure (Hallem et al., 2006). A large fraction of *D. melanogaster* and mosquito ORs confer a decrease in action potential frequency upon odorant exposure (Hallem et al., 2006; Carey et al., 2010), which may be due to an odorant-OR interaction stabilizing a closed ion channel configuration.

In order to better understand the role of ORs in host choice in *An. stephensi* and a non-vector species, *An. quadriannulatus*, we identified ORs which may be involved in human or other animal host choice. We investigated their receptivity to odorants using the *Drosophila* empty neuron system and an previously selected odorant panel (Speth et al., 2021). Several ORs

Although not completely matching, several ORs displayed similar odorant excitation profiles with previously characterized *An. gambiae* ORs. One receptor, *An. quadriannulatus* OR9, was characterized with additional odorants beyond our existing panel and was highly receptive to guaiacol-related compounds.

## **2. Methods:**

### Selection of mosquito odorant receptors for functional characterization

ORs putatively involved in human host choice were predicted based upon an analysis of available antennal transcriptome profiles of Anopheles mosquitoes. At the time this study was conducted, antennal or mouth part transcriptomes of *An. stephensi* were unavailable. The analysis was therefore performed using information from antennal transcriptome studies of *An. gambiae* complex species (Pitts et al., 2011; Rinker et al. 2013a; Rinker et al., 2013b; Athrey et al., 2020; Athrey et al., 2021). It was reasoned that ORs with the largest degree of differential expression between zoophilic species, especially *An. quadriannulatus*, compared to closely related anthropophilic species, especially *An. gambiae* and *An. colluzzii*, were more likely to play a role in host seeking behaviors and contribute to host specificity. It was separately reasoned that ORs which are more highly expressed in non-blood fed female mosquitoes compared to males and blood fed females are more likely involved in mosquito blood feeding behavior. *An. stephensi* homologs of these ORs which were upregulated in 3-5 studies were selected for electrophysiological characterization, with the exception of AsOr76. AsOr76 is absent from the *An. quadriannulatus* genome due to a gene loss event (Table 1). *An. quadriannulatus* ORs which may be responsible for zoophilic host preferences were also chosen for further study (Table 1).

### Mosquitoes

*Anopheles stephensi* colonies (STE2, MRA-128 strain, obtained from MR4, origin: Delhi, India) were maintained at 28 °C and 70% relative humidity on a 16:8 photoperiod (light:dark) in a dedicated insectary. First instar larvae were counted into rearing pans. The larvae were fed daily on a ground fish food diet (Tetramin®, Melle, Germany) until pupation. Adult mosquitoes were provided with water and 10% sucrose solution *ab libitum* for regular colony maintenance. *An. quadriannulatus* colonies (SANGWE, MRA-1155, obtained from BEI resources, origin: Sangwe, Zimbabwe) were similarly maintained.

#### RNA extraction and cDNA library preparation

RNA was extracted from either *An. stephensi* or *An. quadriannulatus* 5 day old adult females maintained on 10% sucrose. Pools of 50 dissected female heads were collected 1-2 h prior to scotophase into 500 µl Trizol. The heads were ground with plastic pestles to prepare a tissue homogenate. A Direct-zol RNA Miniprep Kit (Zymo Research, Irvine, CA, USA) was used for the RNA extractions. RNA samples were treated off-column with DNase I (Ambion, Austin, Texas, USA) for 30 min at 37 °C, otherwise the manufacturer's protocol was followed. The RNA quality was assessed using a Thermo Scientific NanoDrop Bioanalyzer. cDNA libraries were prepared with extracted RNA as template using Superscript IV (Invitrogen, Carlsbad, CA, USA), following the manufacturer's instructions. The cDNA was diluted 1:5 in Ultrapure H<sub>2</sub>O prior to use in PCR amplifications.

#### Cloning

OR sequences were identified by using the tBLASTn function in Vectorbase using the OR sequence from either *An. stephensi* or *An. quadriannulatus* and annotated *An. gambiae* ORs as queries (Giraldo-Calderon et al., 2015). OR annotations in this study are therefore consistent with the *An. gambiae* annotations. OR gene transcripts were amplified using Phusion DNA

polymerase (Phusion Green HSII, ThermoFisher Scientific, Waltham, MA, USA). Primers were designed with restriction site overhangs for each OR, either EcoRI or SACII for forward primers and either XbaI or XhoI restriction sites for reverse primers (Table 2). QIAquick Gel extraction Kit (Qiagen) and Zymoclean Gel DNA extraction kits were used to extract electrophoretically separated PCR products matching predicted open reading frame lengths for the ORs.

Purified PCR products were digested with the respective restriction enzymes (purchased from New England Biolabs) in Cutsmart buffer (New England Biolabs, Ipswich, MA, USA) at 37°C for 2 h, followed by heat inactivation by incubating at 65°C for 20 min. The pUAST vector was digested with respective primer pairs following the same protocol. Ligation reactions were performed by mixing 20 pmol of digested pUAST with 80 pmol of digested insert PCR products with corresponding restriction enzyme overhangs, and incubating with T4 DNA ligase (New England Biolabs) at room temperature for 1 hour. Ligase was inactivated by incubation at 65°C for 10 min. The ligation products were used to heat shock transform either One Shot chemically competent cells or Top10 cells (Invitrogen) by incubation for 30 s at 42°C. After heat shocking, 250 µl of room temperature S.O.C. medium was added to the cells, which were then shaken at 200 rpm, at 37°C for 1 hour. Next, the cells were pelleted, resuspended in 70 µl S.O.C. media, and spread on agar plates with 50 mg/µl ampicillin, warmed to 37°C. Colonies grown overnight were screened for OR open reading frame inserts using PCR. Positive colonies were grown overnight in 5 ml aliquots of LB broth supplemented with 100 mg/ml ampicillin, on a cell shaker at 225 rpm and 37°C. pUAST plasmids containing inserts were extracted from transformed cells using the Qiagen Plasmid Midi Kit (Qiagen, Germantown, MD, USA).

#### D. *melanogaster* stocks and transgenic lines

Each of the ORs characterized in this study was expressed in the *Drosophila melanogaster* empty neuron system (Dobritsa et al., 2003; Hallem et al., 2004). *D. melanogaster*

embryos were transformed by injecting pUAST containing the respective OR inserts (Rainbow Transgenic Flies, Inc., Camarilla, CA, USA). Transformed lines were crossed into either w1118; sco/cyo;Tm6/mkrs or w1118;sp/cyo;Tm6/Tm2 balancer lines and screened for UAS-OR insertions on the third chromosome. The flies were crossed into the  $\Delta$ halo line ( $\Delta$ halo/cyo;22a-Gal4/mkrs flies were kindly provided by Dr. Anandasankar Ray, University of California, Riverside) to generate  $\Delta$ halo/cyo;UAS-OR lines, which were again crossed into the  $\Delta$ halo line. The  $\Delta$ halo/ $\Delta$ halo;22a-Gal4/UAS-OR offspring were selected for use in electrophysiological studies of OR function.

### Electrophysiology

Single unit extracellular recordings in the *Drosophila* empty neuron were performed on 4–14 day old  $\Delta$ halo/ $\Delta$ halo; UAS-OR/22a-Gal4 flies. The methods were adapted from previously described studies (Dobritsa et al., 2003; Mathew et al., 2013). For all recordings, the ground electrode, a pulled glass capillary pipette filled with Ephrussi and Beadle solution (Ashburner, 1989), was slipped over an AgCl coated silver wire and placed into the fly's right eye. The recording electrode, a tungsten microfilament, was placed into direct contact with an ab3 sensilla. The signal was passed through an Iso-DAM8a impedance amplifier (WPI), with the high-pass filter set to 100 Hz and the low-pass filter set to 3 kHz. The signal was digitized at 10,667 Hz using a Syntech IDAC4 Intelligent Data Acquisition Controller. Odorants were diluted 1:100 v/v in paraffin oil and were mobilized with clean, humidified air directed toward the fly antenna at 1.4 L/min (37.5 mL/s). Further dilutions were prepared in paraffin oil via serial dilution for the AqOr9 characterizations. During stimulus presentation, a 0.5-s pulse of 3.75 mL/s amplitude was directed through the shaft of a Pasteur pipette containing 50  $\mu$ L of 1:100 diluted (v/v) odorant pipetted onto a 13-mm Whatman filter paper placed inside the Pasteur pipette. Controlled odor pulses were delivered through the odor cartridges using a Syntech stimulus controller (CS-55).

Odor cartridges were used no more than three times prior to replacement with a new cartridge. Odorants were presented to flies in sets of up to 10 odorants, in addition to solvent control and paraffin oil (PO), while recording from a single fly sensilla. The odorant presentation order was randomized between recordings from single sensilla. Up to three ab3 sensilla per fly were used for recordings. The responses were stored and characterized using Autospike 32 software (Syntech). Action potentials were counted manually over the 500 ms period when the odorant pulse passed the antenna. The average solvent response to paraffin oil was subtracted from the reported action potential firing rates. The odorant panel was previously described (Speth et al., 2021).

#### Protein alignments and structural predictions

OR transmembrane domains were predicted using the TOPCONS consensus membrane topology prediction tool (Tsirigos et al., 2015), except for Or76 transmembrane domain 7. The Or76 transmembrane domain consists of two short alpha helical domains predicted using PSPIRED biotools (Buchan et al., 2013). Or76 transmembrane domain 7 was further predicted through primary structure alignments using other Anopheles OR proteins as references.

### **3. Results:**

#### Activity profiles of two inhibitory ORs, *An. stephensi* Or76 and Or45

Within Anopheles genomes, most ORs cluster within groups of 2-9 genes (Fox et al., 2002; Karner et al., 2015). Or76 belongs to a gene cluster which also includes the odorant receptors Or75 and Or77. Neither Or75 nor Or76 homologues are present in *An. quadriannulatus* genome (Neafsey et al., 2015). We identified both Or76 and Or77 homologs in the *An. stephensi* genome, which suggests that at least one cluster member was lost in the *An. quadriannulatus* lineage. Ors75/76/77 are very similar structurally, with only a few conservative amino acid

substitutions separating the predicted primary structures (Figure 2). Therefore, it was considered that Ors 75, 76 and 77 are likely to have similar functions given the very small degree of structural separation. *An. stephensi* Or76 exhibited significant structural differences from the respective *An. gambiae* Or76 (Figure 2), although many of the primary structure differences were outside of the regions most likely to influence odorant specificity. Important structural differences with respect to *An. gambiae* homologues included non-conservative substitutions of 6/38 amino acids in the EC loop 2 region, which is likely to be an area of odorant binding (Figure 2; Hughes et al., 2014). There is also a section of 9 non-conserved residues in the cytosolic loop between transmembrane domains 4 and 5, however this region exhibits substantial differences in the primary structure for each of the ORs we have modelled (Figure 2).

AsOr76 exhibited inhibitory responses in the ab3A neuron upon exposure to aromatics, especially the cresols (Figure 1, Figure 3A). AsOr76 also inhibited the heterologously expressing odorant sensory neuron (OSN) in response to thiazoles (Figure 1). Propyl acetate, 2-pentanone, and 1-hexanol each resulted in significant inhibition of the ab3A neurons expressing AsOr76. However, excitatory and inhibitory responses of AsOr76 were varied for molecules with similar structures within this group. Many short chain (4-8C) length alcohols and ketones, including propyl acetate and 2-pentanone, strongly excite the ab3B neuron (Figure 3), which expresses *D. melanogaster* Or85b odorant receptor (Hallem et al., 2004; Hallem et al., 2006).

Or45 is more highly expressed in non-blood fed female mosquitoes and has increased expression in anthropophilic species (Table 1). *Anopheles stephensi* Or45 exhibited several structural differences compared to the *An. gambiae* homologue AgOr45, including high substitution rates in transmembrane domains 1-3 (Figure 2). Also notable are non-conservative amino acid substitutions for three out of 42 residues in the extracellular loop 2 region (Figure 2B). Or45 homologues have fewer substitutions in the cytosolic portions of transmembrane domains 4-7, and the cytosolic loop between Tms6-7. This is a region of high structural

conservation among ORs known as the anchor domain (Butterwick et al., 2018). The anchor domain interacts with orco in the formation of functional odorant receptor heterotetramers (Butterwick et al., 2018).

Neurons expressing AsOr45 were inhibited primarily in response to phenols, including all three cresols, and indole was also inhibitory (Figure 1). Or45 also exhibited some responses to short chain alcohols and ketones, although responses were lesser in magnitude than those observed for AsOr76.

Both Or76 and Or45 expressing neurons exhibited some inhibitory responses to short (4-8C) chain length alcohols and ketones (Figure 1). It was clear from the electrophysiological recordings that inhibition of ab3A neuron expressing either AsOr45 or AsOr76 with aromatics resulted in increased B neuron spontaneous activity (Figure 3). This result is consistent with a pattern of inhibition of neighboring ORNs previously observed in *D. melanogaster* (Su et al., 2012). While it cannot be ruled out that AsOr76 has an independent sensory function in detecting 4-8C alcohols and ketones, the results for odorants which strongly activate the *D. melanogaster* ab3B neuron should be interpreted with caution. It is also notable that Ab3a neurons expressing the inhibitory receptors *An. stephensi* ORs 45 and 76 had higher spontaneous firing frequencies than did neurons which responded to odorants with increased activity.

#### AsOr41, AsOr60 and AqOr63 are inactive in the empty neuron system

Anopheles Ors 41 and 60 have greater transcript abundance in the antennae of female *An. gambiae* (Pitts et al., 2011) and are also present in greater abundance in the antennae of females prior to blood feeding (Table 1) (Rinker et al., 2013). Similarly, *An. quadriannulatus* ORs 18, 63, and 74 had greater transcript abundance in the antennae of the zoophilic species in comparison to two important vectors of human malaria, *An. gambiae* and *An. colluzzii* and were

also chosen for characterization (Rinker et al., 2013; Athrey et al., 2020). However, we were unable to clone predicted AqORs 18 and 74, despite multiple attempts.

None of AsOr41, AsOr60 and AqOr63 exhibited significant responses in the empty neuron system (Figure 1). The range of responses for each of these receptors was -8.5 to 22 action potentials per second, which were within the margins of error (95% C.I.) for the receptor responses. In addition, ab3A neurons expressing the receptors exhibited very low spontaneous activity. Averaged spontaneous firing rates of ab3A neurons expressing mosquito odor receptors were 0 /sec (n=9) for AqOr63, 1.3 /sec (n=17) for AsOr41, and 2.2 /sec for AsOr60 (n=37). In comparison, ab3A neurons expressing mosquito odor receptors which showed strong odorant induced responses had much higher frequency spontaneous action potentials: 25 /sec (n=32) for AsOr76, 30 /sec (n=36) for AsOr45, 12.5 /sec (n=12.5) for AqOr9 and 9 /sec (n =31) for AsOr8.

#### AqOr9 is highly sensitive to guaiacols

Or9 transcripts are significantly enriched in the antennae of *An. quadriannulatus* in comparison to the anthropophilic *An. gambiae* (Rinker et al. 2013). AqOr9 expression has also been detected in the mouthparts of *An. quadriannulatus*, but is absent from the mouthparts of the predominant vectors of human malaria including *An. coluzzii*, and *An. stephensi*. (Athrey et al., 2021; Speth et al., 2021). However, Or9 transcripts are present in the antennae of both *An. coluzzii* and *An. gambiae* (Rinker et al., 2013; Athrey et al., 2020).

Among the original 48 odorants tested, AqOr9 responses were greatest for aromatics, in particular 2-ethylphenol and 2-propylphenol, as well as thiazoles containing methyl branches or esters (Figure 1). It was unclear from this data what ecological roles AqOr9 might serve. Based on the structures of odorant molecules which most strongly activated AqOr9 expressing ab3A neurons in the initial odorant induced activity screen (Figure 1), we selected a second panel of VOCs. Odorants included in the second panel were selected to meet two separate criteria, the

first being structural similarity to activating molecules identified in the first panel and the second being presence in natural odor sources which are important to mosquito ecology. Towards this end, we selected primarily phenolics with one or two carbon side chains, as well as polar side chains, which may influence the OR-odorant binding affinity. Side chain position was also considered. Also selected were thiazoles with methyl groups and polar side chains in differing positions. Molecules included in the second odorant panel were also chosen for presence in plant odors which are attractive or repellent to mosquitoes. Other odorants were added to expand the structural diversity of the panel. Altogether, the second odorant panel includes odorants which are present in floral scents, essential oils, products of microbial fermentation, or smoke (Figure 4A).

From this second panel, activity was highest for guaiacols, xyleneol, and methylphenols. Molecules with structural similarities for the most activating compounds, including 4-ethylguaiacol and 4-methylguaiacol, were included in a third odorant panel. The combined results for AqOr9 expressing ab3A neuron responses to reiteratively selected odorants are presented in Figure 4. All molecules which excited neurons at greater than 100 action potentials/s were tested at more dilute concentrations. At  $10^{-4}$  (vol./vol.) dilutions of odorants in paraffin oil (equivalent to ~5 nanograms per assay), neurons expressing AqOr9 still responded strongly to 4-ethylguaiacol, guaiacol, xyleneol, and 2-methoxy-4-methylphenol (4-methylguaiacol), but not 2-ethylphenol (Figure 4B). At dilutions of  $10^{-5}$ , or ~0.5 nanograms, the guaiacols and xyleneol still elicited significant neuronal responses at (Figure 5). Guaiacol and 4-ethylguaiacol excited AqOr9 expressing neurons at dilutions as low as  $10^{-6}$  (~50 picograms of odorant), although not all neurons were stimulated at these concentrations (Figure 5).

#### **4. Discussion:**

### Identification of *An. stephensi* host seeking genes

This study's original OR gene selection criteria was based in part on the hypothesis that sex specific differences in OR abundance could result from positive selection for female ORs involved in sex specific behaviors, especially host seeking or ovipositioning. However, for the ORs tested, we did not observe enriched detection of animal host odorants, including human odorants, for the ORs characterized in our study (Figure 1). It should be noted that *An. gambiae* females have more antennal transcripts for all but a few odorant receptors than males (Pitts et al., 2015). Most differences in Or transcripts between sexes are likely a result of pronounced sexual dimorphism of the *Anopheles* antennae. OSNs are present on 12 segments of the antennae of female *Anopheles* mosquitoes and only the 2 distal most male segments, and females have more than twice as many OSNs on the antennae (Riabinina et al., 2016). For these reasons, differences in antennal OR transcripts between sexes may not be particularly informative as a predictor chemosensory gene involvement in sex specific behaviors, unless scaled to offset this sexual dimorphism.

At the beginning of this study, it was unclear how well OR function would be conserved between taxa as distantly related as *An. gambiae* complex species and *An. stephensi*. It was predicted that ORs which were enriched in the antennal transcriptomes of anthropophilic versus zoophilic species would more likely play a role in host detection. *An. stephensi* ORs were selected on this basis from an analysis of transcriptomic studies each performed on *An. gambiae* complex species. However, *An. stephensi* and *An. gambiae* evolved anthropophily independently (Neafsey et al., 2015). It is equally possible that gene gain and loss events, or different expression patterns within OSNs, or even different neuronal connectivity of OSNs and other neurons involved in olfactory processing could contribute to convergent host preference. Out of seven odorant receptors characterized, 5 of which were enriched in the antennae of anthropophilic vector species of the *An. gambiae* complex and 2 of which were enriched in the zoophilic *An.*

*quadriannulatus*, only one receptor, *An. stephensi* Or8, which was described in a previous study (Speth et al., 2021), had an activity profile consistent with the detection of human and animal odorants (Figure 1).

Despite the failure to predict host detecting *An. stephensi* odorant receptors from transcriptome datasets, the results of this study do allow for a cross-species comparison of insect odorant receptor function between two important malaria vectors. Further, a previously unreported mosquito odorant receptor chemical sensing activity was discovered for AqOr9.

#### Function of two inhibitory odorant receptors, Or45 and Or76

Neurons within a shared sensilla may influence adjacent neuron activity by changing ionic concentration of the sensillar lymph and thus influencing transmembrane potential of the adjacent neuron (Vermeulin & Rospars, 2004). This interaction is known as ephatic coupling. Ephatic coupling may play a role in the observed depression of ab3A neurons expressing AsOr76 with short chain length alcohols and ketones, each of which results in ab3B neuron excitation (Figure 3) (Su et al., 2012).

Several *Drosophila spp.* and *Anopheles spp.* ORs confer primarily inhibitory responses in ORNs to many odorants, and the proportion of ORs conferring predominantly inhibitory responses is greater for *Anopheles* than *Drosophila* (Hallem et al., 2006; Carey et al. 2010; Mathew et al., 2013). In *D. melanogaster* larvae, odorants which inhibit several receptors tend to induce repellent behavior (Kreher et al., 2008).

On the *D. melanogaster* antennae, ORN pairings are conserved between sensilla of the same type (Hallem et al., 2006). In *D. melanogaster*, ephatic coupling between neurons plays a role in behavioral regulation through interactions between paired neurons which antagonistically contribute to the same behavior (Wu et al., 2021). This arrangement effectively reduces upstream computational costs in the *D. melanogaster* central nervous system (Wu et al., 2022). Sensilla on

the mosquito antennae show the same ordered arrangements of ORNs (Qiu et al., 2006), which may have evolved as an efficient information processing system. An information theoretical model of olfactory coding predicts an increase in coding capacity with inhibitory odor-receptor interactions, assuming that neurons maintain a non-zero basal activity (Qin et al., 2019). In this model, with higher basal neuronal activity, the predicted number of inhibitory interactions which maintains maximum entropy is increased (Qin et al., 2019). Therefore, it is likely that the inhibitory ORs 45 and 76, as well as other inhibitory odorant receptors, play a role in increasing the efficiency of odor coding in flies and mosquitoes. Studies are increasingly showing that ephatic coupling contributes to mosquito behavior, and is likely selected for (Wu et al., 2021). However, for functional characterization of ORs, ephatic coupling poses a challenge. A different empty neuron system, or a sensilla devoid of endogenous activity altogether, may be required for full functional characterization of inhibitory ORs in the future, which would allow for more accurate structure function comparisons.

#### Conservation of OR gene function across species

*Anopheles* ORs have a high rate of synonymous to non-synonymous mutation, which is characteristic of strong positive selection. Both ORs and GRs have among the highest evolutionary rates among gene families in *Anopheles* mosquitoes (Neafsey et al., 2015). Other than an expansion of the OR gene family within the *An. gambiae* species complex, members of which have about 15 more OR genes than most other Anophelines, gene gain and loss among the *Anopheles* chemosensory receptors (ORs, GRs and IRs) is actually lower than for most genes amongst the Anopheline genome (Neafsey et al., 2015). This information, paired with the observation that closely related *An. gambiae* complex sibling species exhibit vastly different blood feeding preferences, has led to two hypotheses proposed to explain chemosensory gene contributions to differences in species-specific behaviors. One hypothesis is that functional

divergence of ORs and GRs contributes to differences in host seeking (Neafsey et al., 2015). A second hypothesis is that differential transcriptional regulation of gene orthologues contributes to host seeking differences (Neafsey et al., 2015). A third hypothesis, which was not addressed in this study, is that differences in neural connectivity are responsible for species-specific behaviors. None of these hypothesis are not mutually exclusive.

In only a few cases have functional studies allowed cross-species comparisons of functional conservation of ORs and GRs amongst Anophelinae. The best studied example of a conserved sensory function between mosquitoes is that of the capitata peg, which is the only type of chemosensory sensilla located on the mouthparts of both *Anopheles* and *Aedes*. Within the capitata peg is an OSN which expresses the CO<sub>2</sub> receptor genes, and this neuron's function as a CO<sub>2</sub> sensor is conserved between *Aedes* and *Anopheles*. Interestingly, a second neuron in the capitata peg which is highly sensitive to 1-octen-3-ol, a component of human sweat odor, is also conserved between divergent mosquito species, including *Aedes* and *Anopheles* and even *Toxorhynchites amboinensis*, which do not take blood meals (Liu et al., 2007; Bohbot et al., 2013; Zhou et al., 2014; Dekel et al., 2016; Speth et al., 2021). A different study identified a conserved indole responsiveness between *Ae. aegypti* OR2 and *An. gambiae* OR10, which exhibit a high degree of sequence conservation (for ORs) and are likely orthologues (Bohbot et al., 2010). These results suggest that at least for the most highly conserved ORs, functions are conserved between Anophelinae and Culicinae. The extent to which functional conservation applies to the many more divergent ORs remains unknown.

The *An. gambiae* OR repertoire has been extensively characterized and is currently the best studied of any mosquito species, with the activity of over 50 ORs characterized across a wide range of odorant molecules (Wang et al. 2010; Carey et al., 2010). However, for other mosquito species, few ORs have been characterized, and there is an overall dearth of information allowing for interspecies comparison of OR functions, other than for OR8, amongst gene orthologues (Liu

et al., 2007; Speth et al 2021). In the current study, *An. stephensi* and *An. quadriannulatus* ORs were selected to address host selection and functional conservation of OR function.

Two of the *An. stephensi* ORs characterized in this study had partially conserved functions in comparison with *An. gambiae* orthologues. *An. stephensi* Or45 and Or76 each inhibit the expressing ORN while exposed to aromatics, which was a similarly identified activity for the corresponding *An. gambiae* ORs (Carey et al., 2010). In particular, AgOr76 exhibits inhibitory responses to both phenols and heterocyclics, with 4,5-dimethylthiazole resulting in the greatest depression of ab3A activity (Carey et al., 2010). This response pattern is also characteristic of AsOr76 (Figure 1). The *Anopheles stephensi* Or45 response was only partially conserved for heterocyclics. *Anopheles stephensi* Or45 did not have inhibitory responses to thiazoles which are characteristic of the *An. gambiae* Or45 (Carey et al., 2010). The *D. melanogaster* ab3B neuron is not independently responsive to either aromatic or heterocyclic molecules (Hallem et al., 2004).

For other types of odorants, responses between *An. stephensi* Ors 45 and 76 are dissimilar. Most noticeably, activity differed between *An. stephensi* and *An. gambiae* Or76 in response to short (4-8C) chain length alcohols, aldehydes and ketones (Figure 1; Carey et al., 2010). The interspecies activities of Or45 are also most dissimilar for the short chain alcohols, aldehydes and ketones, many of which strongly excite the neighboring ab3B neuron (Figure 3). Functional characterization of inhibitory ORs in a neuron with activity independence from neighboring neurons may be necessary for full spectrum OR-odorant function comparisons. Neuronal inhibition of ORNs does not have the same dynamic range as activation. Characterizing ORs in an ORN which is independent of odorant induced activation of a neighboring neurons may be more important for ORs with inhibitory activity.

Other interspecies OR orthologue activity comparisons can be made for Or8. Odorant receptor 8 orthologues have been characterized for *An. stephensi* and *An. gambiae*, as well as

non-Anophelines including *Toxorhynchites amoboensis*. *T. amoboensis* Or8 retains a high sensitivity to 1-octen-3-ol, but lacks responsivity to similar chain length alcohols and aldehydes which strongly activate both *An. stephensi* and *An. gambiae* Or8 (Liu et al., 2011; Dekel et al., 2016; Speth et al., 2021). Activity comparisons between Or orthologues from different mosquito species therefore suggest a pattern of partial functional conservation. Specific, primary sensing functions may be more conserved. Secondary sensory functions of ORs may be more divergent as mutations accumulate. It should be noted that for studies which allow functional comparisons, odorant-OR interactions have been screened at odorant concentrations unlikely to be found in nature, so the ‘true’ degree of conservation of OR activities may be more highly conserved for ecologically important odorants.

#### AqOr9 function

In our study, *An. quadriannulatus* Or9 had highly similar responses to the *An. gambiae* Or9 to 28 odorants, with a correlation coefficient of 0.94. This was not too surprising given how closely related the two species are. However, by selecting similarly structured VOCs to Or9 activating molecules in the original screen we were able to identify a previously unknown guaiacol sensing function of this odorant receptor. The magnitude of responses elicited by guaiacol compounds, even at low concentrations, was much greater in comparison to the previously identified most activating volatiles, including 2-ethylphenol (Figure 4). With this new data, a reinterpretation of the odor space and functional role of this AqOr9 is in order.

AqOr9 activating odors were not enriched for odorants which are common constituents of human and animal odors. However, AqOr9 expressing neurons were strongly activated by phenols and thiazoles. In light of this information, we set out to further determine the function of AqOr9 by selecting a new panel of odorants with structural similarities to the activating odors. Given the strong excitation of AqOr9 expressing fly neurons to aromatic molecules, we

anticipated that AqOr9 might play a role in the detection of plant odors. Odorant components of floral scents, including VOCs produced by plants which are attractive to *Anopheles* mosquitoes (Nikbakhtzadeh et al., 2014), were included in a second odorant panel. Also included in the second odorant panel are products of microbial fermentation, a few of which are indicative of food nutritional quality and are attractive to fly larvae (Dweck et al., 2015). We selected phenolics with different polar side chains to investigate the effects of anionic charge on odorant structure- AqOr9 function relationships. We also selected phenols and thiazoles with side chains in different positions to assess the stereochemical effects. Structures were varied to increase the volume of odor space sampled.

AqOr9 expressing neurons were most strongly activated by guaiacols (Figure 4), which may have several different sources in nature. Guaiacol is one of the main components of lignin and is released during lignin combustion (Asmadi et al. 2011). Further pyrolysis of guaiacol results in several products which are primarily phenols, cresols and xylenols (Asmadi et al., 2011). Interestingly, the AqOr9 responded strongly not only to guaiacol, but to other primary products of guaiacol pyrolysis including 2,4-xylenol, o-cresol and 4-methylguaiacol at lower concentration thresholds (Figures 4, 5). Among the most excitatory odorants tested at the lower concentrations, 4/5 odorants eliciting strong excitation are products of lignin combustion (Figure 5; Asmadi et al., 2011).

A different source of AqOr9 activating odors is microbial fermentation. 4-ethylguaiacol is detected by a *D. melanogaster* Or71a, and is utilized in fruit flies for proxy detection of dietary antioxidants produced by *Brettanomyces* yeast (Dweck et al., 2015). *Drosophila melanogaster* exhibit a feeding preference on fruits inoculated with *Brettanomyces*. In return, fly larvae feeding on medium with these yeast have increased survival when also ingesting molecules which induce oxidative stress, including paraquat (Dweck et al., 2015). 4-ethylguaiacol can be produced through yeast fermentation of different fruits and is best studied in the context of aging wine. The

production of 4-ethylguaiacol by *Brettanomyces* in aging wine barrels is associated with a smoky aroma and is often co-produced with 4-ethylphenol, which is the cause of the often undesirable ‘Brett character’ (Suarez et al., 2007).

Guaiacol and many of its derivatives are phytochemicals and important components of plant oil scents. 4-allylguaiacol is a primary component of clove oil scent, and other guaiacol derivatives produce spicy scents (Ghadirasli et al., 2021). Guaiacol and related odorants could therefore be deterrent to non-specialist insects reliant upon plant sources for sustenance, such as mosquitoes. Interestingly, guaiacol and guaiacol derivatives have previously been identified as tsetse fly repellents, and 4-methylguaiacol (2-methoxy-4-methylphenol) is a superior *Glossina pallidipes* repellent (Saini et al., 2007). Conversely, guaiacol is a product of microbial degradation of lignin, a primary structural biopolymer component of plants, and could potentially be utilized by female mosquitoes during ovipositioning. More data is needed to uncover the relationships of guaiacol and its derivatives to mosquito behavioral ecology.

Flies of the genus *Microsania*, also known as smoke-flies (family Platypezidae), are attracted to smoke released by brushfires (Klocke et al., 2011). While *Microsania*, could be utilizing CO<sub>2</sub> to detect brushfires, the flies also oviposit around burned wood and soil (Klocke et al., 2011). Smoke flies may be utilizing the same molecules released by lignin pyrolysis detected by AqOr9 for navigation.

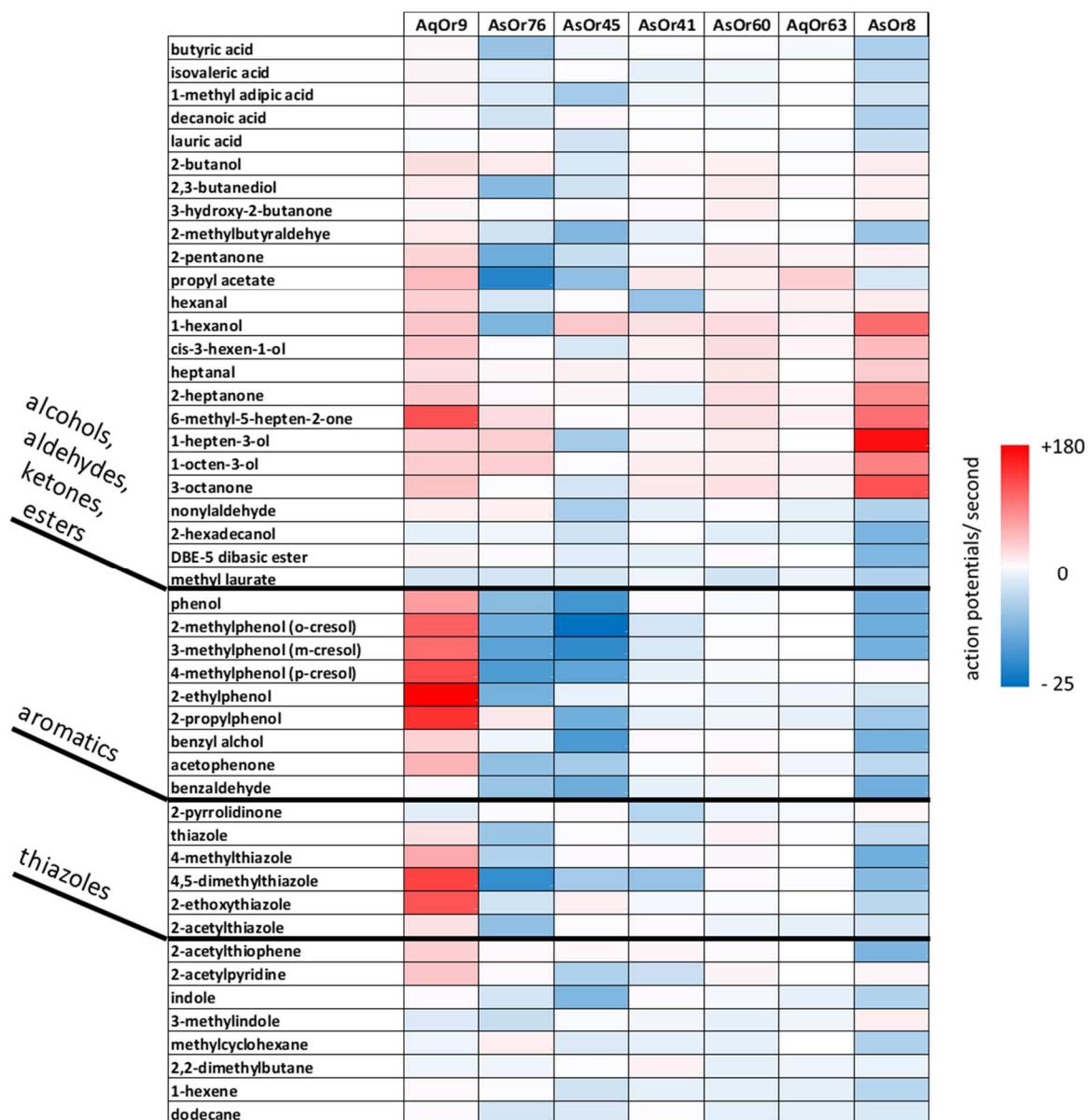
The implications for a mosquito odor detector highly sensitive to smoke odorants remains unclear. Since the earliest periods of anthropoid fire use, *Anopheles* mosquitoes have evolved anthropophilic feeding behaviors several times (MacDonald et al., 2021; Kamali et al., 2012; Thawornwattana et al., 2018). It is worth investigating whether mosquitoes are attracted to or repulsed by guaiacols and other odorants detected by AqOr9, and whether there are differences in attraction dependent upon physiological state or between anthropophilic and zoophilic species.

**Table 1. Gene expression patterns of odorant receptors from anthropophilic and zoophilic species characterized in this study.**

Or gene	Reference sequence gene ID	Greater relative abundance: <i>An. gambiae</i> , <i>An. colluzzii</i> or <i>An. quadriannulatus</i> ( <b>A1</b> , <b>A2</b> , <b>R</b> )	Increased transcripts: prior to bloodmeal, or in female mosquitoes ( <b>B</b> , <b>S</b> )
AqOr9	AQUA010654-RA	<i>quadriannulatus</i>	
AsOr41	ASTE015972-PA	<i>colluzzii</i>	Female and pre- blood meal
AsOr45	ASTE002773-PA	<i>colluzzii</i>	Female and pre- blood meal
AsOr60	ASTE008478-PA	<i>gambiae</i>	Female and pre- blood meal
AqOr63	AQUA008657-RA	<i>quadriannulatus</i>	
AsOr76	ASTE000155-PA	Both Or76 and Or75 are absent from <i>An. quadriannulatus</i>	Female and pre- blood meal
<p><b>Study descriptions</b>  <b>A1</b> Athrey et al., 2020: comparison between <i>An. quadriannulatus</i> and <i>An. colluzzii</i> antennal transcriptomes  <b>A2</b> Athrey et al., 2021: comparison of mouth part transcript abundances between <i>An. quadriannulatus</i> and <i>An. colluzzii</i>  <b>B</b> Rinker et al., 2013: blood meal-induced differences in OR transcripts, <i>An. gambiae</i>  <b>R</b> Rinker et al., 2013: interspecies comparison of <i>An. gambiae</i> and <i>An. quadriannulatus</i> antennal chemosensory gene transcripts  <b>S</b> Pitts et al., 2011: sex-specific differences in OR transcripts, <i>An. gambiae</i></p>			

**Table 2. Primers and PCR conditions used to clone odor receptors.**

Odorant receptor		Primer name	Sequence
AqOr9	Forward	AqOr9F1EcoRI	CTAGAATTCACCATGGTTAGGCTCTTCTCCGC
	Reverse	AqOr9R1Xbal	GGCAGTTTTGAGATCGATGACGGATTAGTCTAGATAG
	2-step PCR: 64°C for 30s, 10 cycles; 69°C for 30s, 30 cycles		
AsOr76	Forward	AsOr76F1EcoRI	CTAGAATTCACCATGGTTGTGAAGCGTGCCC
	Reverse	AsOr76R1SacII	GGGCATGGCTAATTTAGCAACCTAACCGCGGTAG
AsOr45	Forward	AsOr45F1SacII/SDA	CTACCGCGGACCATGAAGCAGGTCAGTACGAGTGAA
	Reverse	AsOr45R1XhoI	CCTATTCGTACTTCACCATACTTCATTGACTCGAGTAG
	PCR: 64°C for 30s, 40 cycles		
AsOr41	Forward	AsOr41AF1EcoRI	CTAGAATTCACCATGGCAGCAGTCAAGGAAAGTC
	Reverse	AsOr41AR1XhoI	CTCCATATTTATGATGCTGGAAAACATGTGACTCGAGTAG
	2-step PCR: 61°C for 30s, 10 cycles; 67°C for 30s, 30 cycles		
AsOr60	Forward	AsOr60F1SacII	CTACCGCGGACCATGGAGCTGTTTTTGTCCCAGA
	Reverse	AsOr60R1XhoI	GCTGCAGCTAAAAAATGAAATTGCCTCCTAGCTCGAGTAG
	Touchdown PCR: 72-63°C for 30s (-1°C for each cycle), 10 cycles, 69°C for 30s, 30 cycles		
AqOr63	Forward	AqOr63F1EcoRI	CTAGAATTCACCATGTACCTTACGCAGCTATTCGCCA
	Reverse	AqOr63R1Xbal	CGTTTTTGCTGCAGTTCGCAAAGTAGTCTAGATC
	PCR: 64°C for 30s, 40 cycles		
For all PCRs, per cycle: denaturation was performed at 98°C for 15 seconds, and extension at 72°C for 75s. Specific annealing conditions are noted for each primer pair.			



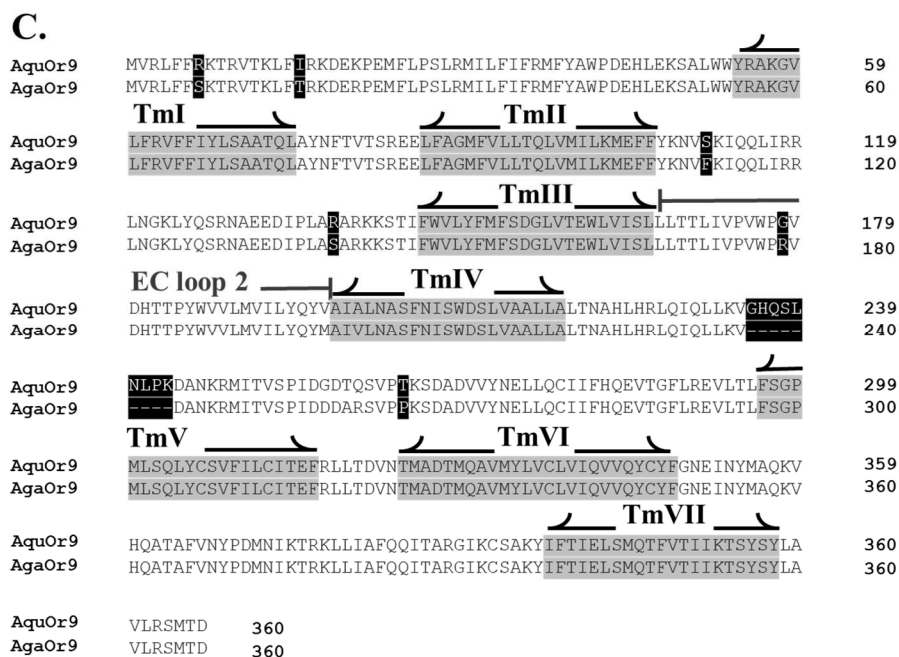
**Figure 1: OR-odorant interaction heatmap.** Alcohols aldehydes and ketones were grouped together and arranged by chain length and similarity. Aromatics, thiazoles, other heterocyclics and nonpolar VOCs were grouped and arranged by structural similarity. The responses represented in the heat map are averages of N=3 to N=9 single sensillum recordings. Only 30 of the odorants were tested for AqOr63.

## A.

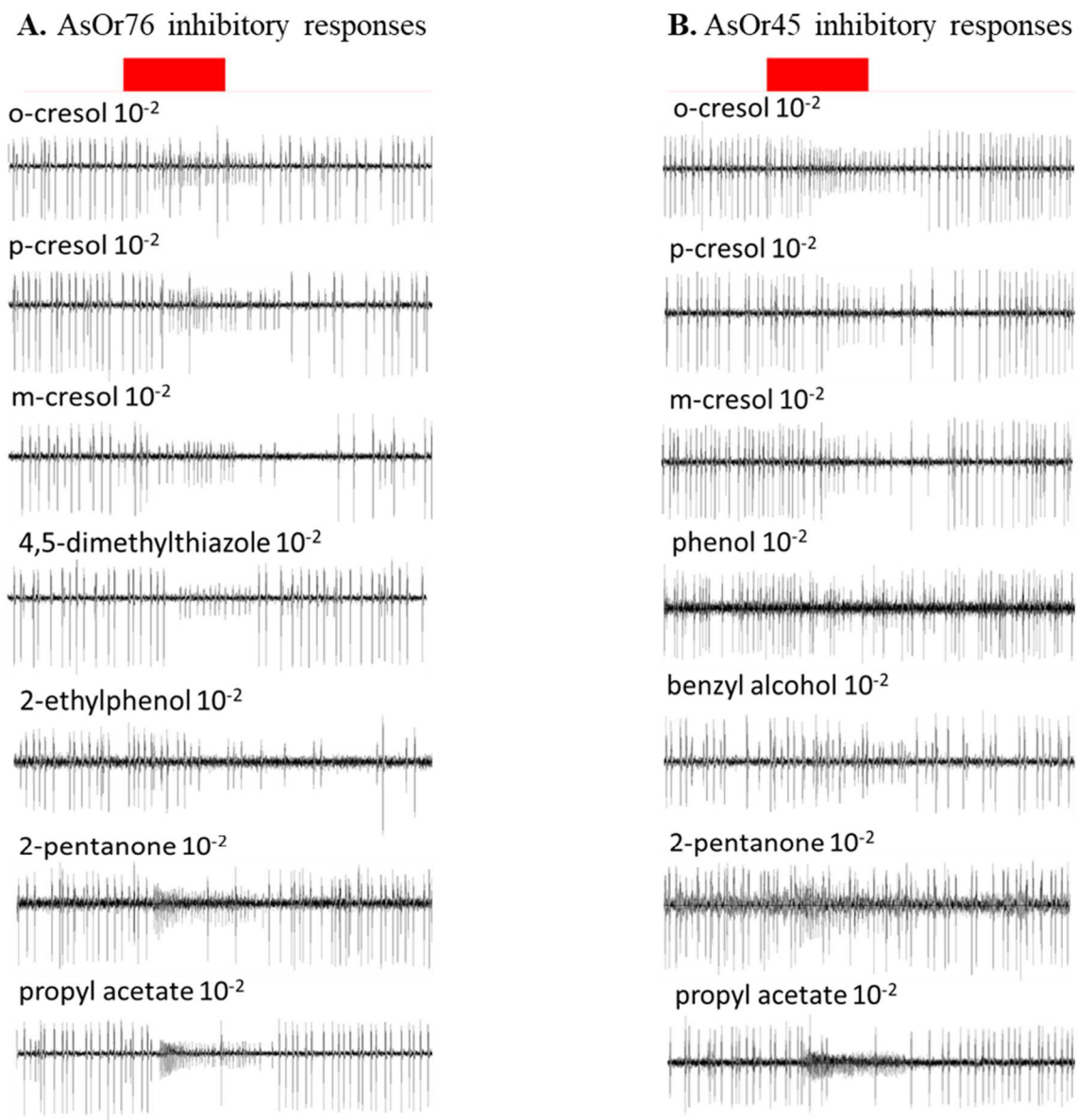
AsOr76	-MVKRALIYSFSLQHHFNVGHPDEHFCLLRCLDVPSPAMLIQRPRSGLEIGIKTLSLS	59
AgOr76	MTVVHRIVSFGYNLLQRHFNVGHPTEQFELLRCLDVPSPAMLLQRPRSNLEVALKTLCLS	60
AgOr75	MTVVHRIVSFGYNLLQRHFNVGHPTEQFELLRCLDVPSPAMLLQRPRSNLEVALKTLCLS	60
	<b>TmI</b> <b>TmII</b>	
AsOr76	VLLTHVVGLEYDLTQCEDIRVAMDIFCMLSLFSSLFARNTCLRQYQSHIIAMERLDANPG	119
AgOr76	VMVAHTIALAYDFSQQMDVRLALDMLCMLSLFVSIILRSTCMRQYLSHIDALDRLERPT	120
AgOr75	VMVAHTIALAYDFSQQMDVRLALDMLCMLSLFVSIILRGTCMRQYLAHINALDRLERPT	120
	<b>TmIII</b>	
AsOr76	FEVGVPYAETIRHRVTIQNNRYLGGWYLVSHSLTIVTYVTQNMAMQGSFVKIITTFPIDLS	179
AgOr76	FRVGTPYADESRRNVALQNSRYLGVALLVMSLTVTMYVTQNMVRENSFVKIITTFPIDLS	180
AgOr75	FRIGTPYADESRRNVALQNSRYLGVALLVMSLTVTMYVTQNMVRENSFVKIITTFPIDLS	180
	<b>EC loop 2</b> <b>TmIV</b>	
AsOr76	GYAPALDITMTQFFYTVAGYGNWAWYHAAQGLIVIVLLRFATAEFRVFLHSLATLDEQIHDR	239
AgOr76	ERAPVLERVADLCYSLVGYVWGWYHGATQLTIIIVLLRYAIEFRVFLHSLATLDDQLRQR	240
AgOr75	ERAPVLERVADLCYSLVGYVWGWYHGATQLTIIIVLLRYAIEFRVFLHSLATLDDQLRQR	240
	<b>TmV</b>	
AsOr76	LLLE-LDGNEEERVVRELLYKHARQHSQILIVVVMHLRAILRISLVHFSFYMIIMAAFMAR	298
AgOr76	REQAQGAPDEERILRELLYEHARHHSQILIVVVTHLRTLRLRNSLVHFFFYMIIVATFMTR	300
AgOr75	REQSQGAPDEERILRELLYEHARHHSQILIVVVTHLRTLRLRNSLVHFSFYMIIVATFMTR	300
	<b>TmVI</b>	
AsOr76	VLIIPGSSSLGMAIPVLVTTIVFFLETFGLCMLVBLVQLNRRVSVNLYGFGWTRYLQYGH	358
AgOr76	VLIIPGSSSFLAIPLLTITTYFFETFGMCMLEMLVQLNRKVVSTSLYGFSWPQYLRYGR	360
AgOr75	VLIIPGSSSFLAIPLLVTTIVFFLETFGMCMLEMLVQLNRKVVSTSLYGFSWPQYLRYGR	360
	<b>TmVII</b>	
AsOr76	SIKPTMMLMIMQANNTKDFSAGGLTTVSAELFAKTCRLVYTMMMGMANLAT	409
AgOr76	TIKPTMMLMIMQANNTKDFSAGGLTTVSAELFAKTCRMIYTMMMGMANMAT	411
AgOr75	TIKPTMMLMIMQANNTKDFSAGGLTTVSAELFAKTCRMIYTMMMGMANMAT	411

## B.

AsOr45	-MKQVSTSEANLERMFALIAKHMEVVLKLNIFKPEWRLSLRTVLVIVATGFMPIILTAFSVN	59
AgOr45	MPRATSNNSAANLERLIVIRISSHMAVVLKLNIDPAWRPTLRFGIVLFLTALVPVYIWQGIK	60
	<b>TmI</b>	
AsOr45	KYYEHLKIVKVECFQACTGAQVFIRSYFYLRQRDQCRQLATEIRRQRISYGVNEHERMEQ	119
AgOr45	VYRTRFETLLEVLVSVAGCGWQMFRRMYFYLRQQDRCRQIVQEVREIORTVYGADRNRMEK	120
	<b>TmII</b>	
AsOr45	LFRFRATERMLMILYRLMYAMYCCSFFFVLGPLIMPDSRKASLPLAFRIPYLPDENLLYWC	179
AgOr45	LFRAGTKRMLLAYRVIHLMYGTSYFFQLGPLIMPDPHKCNLPLALQLFPLPPDRNMVYYC	180
	<b>TmIII</b> <b>EC loop 2</b>	
AsOr45	LNYLHHIFLIVVGIHHLAPIDGIVLALISICTRISALELLNELDGGKITESKWQTEHL	239
AgOr45	INYLHHLLNTIGVFLLLPMDGVLIIVALLNICTRIAALQLLEELDAGLGTWQQT AHL	240
	<b>TmIV</b>	
AsOr45	EPCLDRIIEHLHDKRFAELVSTFEMHFFTFMSICSTICMCLNVIAGQPRNSTYPIIDL	299
AgOr45	DAEALNRIIEHLHDKRFAELVSTFEMHFFSMFVLCFVICMCMNVVARDPRSTLIPFGL	300
	<b>TmV</b>	
AsOr45	ASVQQLFVVCLFGNVLLIVNDRLEPKSIYGIQWYRIVTQKKILFLLANAQTDIVMSAVF	359
AgOr45	ASTCQLFVICMLGNVLYIVSDRLKDSVYGIWYRIVTQKKILFLLANAQTEIVMGAVF	360
	<b>TmVI</b>	
AsOr45	KPVNMTSFVAVCRASYSYFTILH	382
AgOr45	IPVTMTSFVTIIRAAYSYFTILY	383
	<b>TmVII</b>	

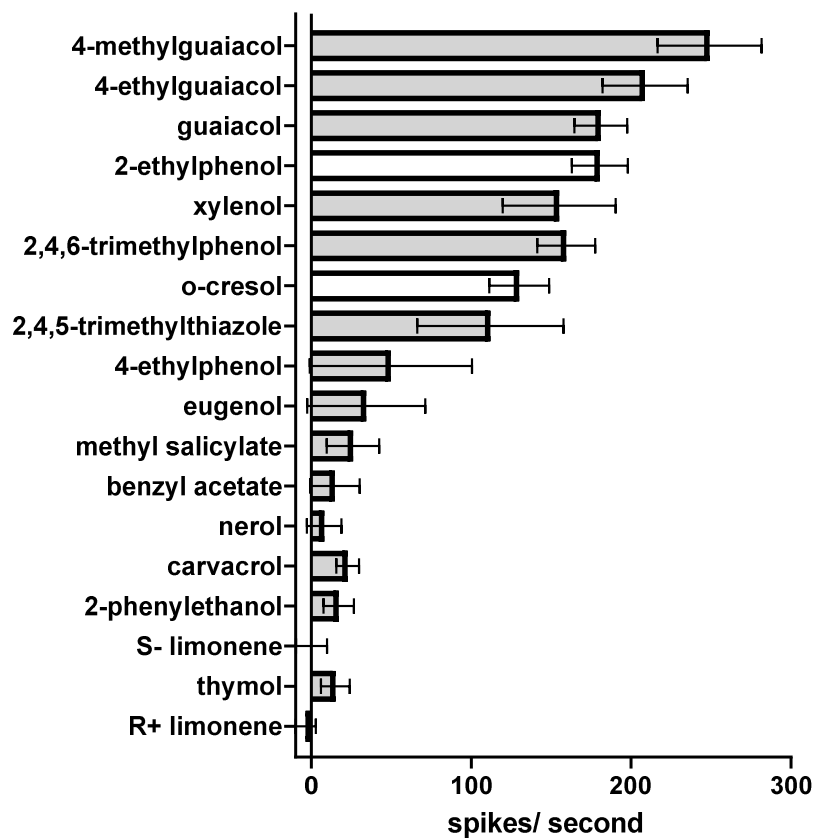


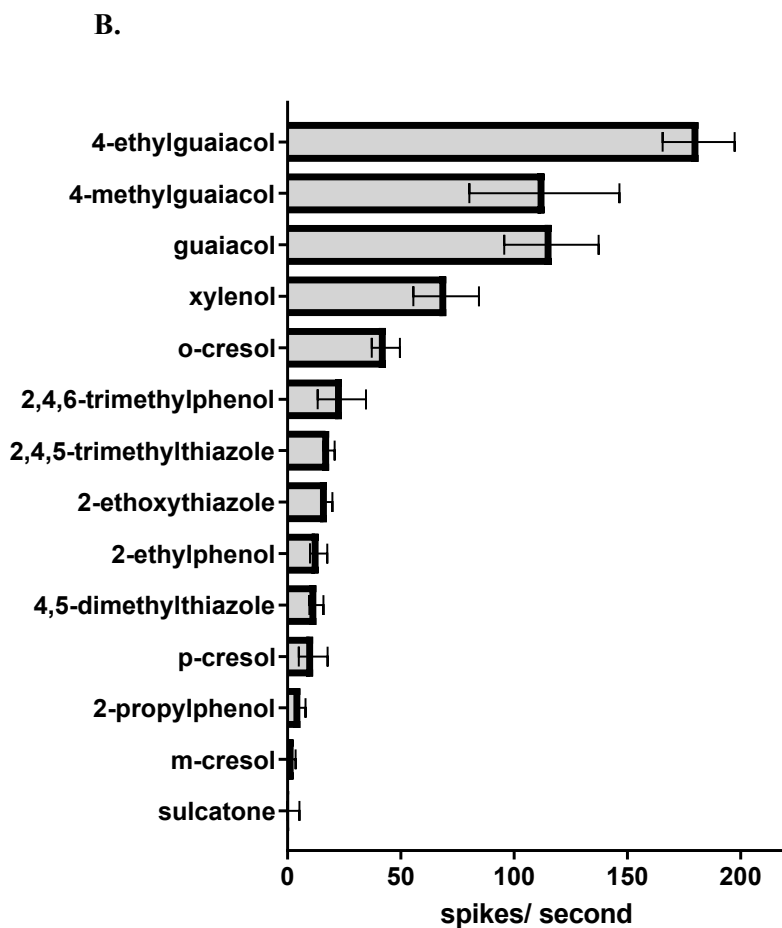
**Figure 2: Primary structure alignments of ORs characterized in this study with previously characterized *An. gambiae* ORs (PEST reference sequence). A) Or76; B) Or45; C) Or9. Black highlights = non-conservative amino acid substitutions or insertion or deletion. Predicted transmembrane domains are labelled TmI-VII and highlighted in gray. EC loop 2 is the second extracellular loop domain, a ligand binding region. The N-terminus is cytosolic.**



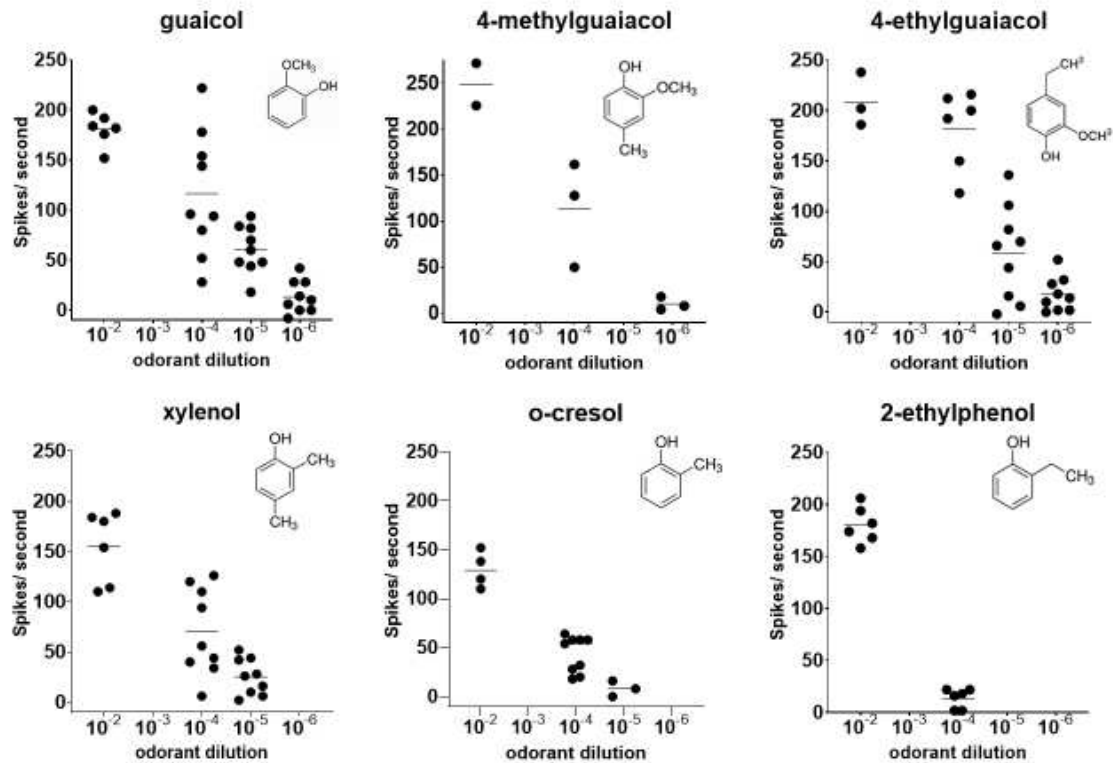
**Figure 3: Single sensillum recording signal traces showing odorant induced inhibitory responses of ab3A neurons expressing A. AsOr76, or B. AsOr45.** The greater amplitude spikes are ab3A neuron action potentials, and the smaller amplitude spikes are ab3B neuron action potentials. Note that the ab3B neuron exhibits some excitation in response to ab3A neuron inhibition induced by phenolics. The ab3B neuron, which does not respond independently to phenolics, is directly activated by 2-pentanone and propyl acetate (Hallem et al., 2004; Hallem et al., 2006). The responses shown are the median responses for each OR-odorant pairing, except for the AsOr45 propyl acetate response. The red bar indicates the odorant pulse, which reaches the antennae about 0.2 seconds after the start of the pulse. Signal traces are shown for 2 second periods.

A.





**Figure 4: Odorants selected from reiterative process strongly activate AqOr9 expressing ab3A neurons.** **A.** AqOr9 responses to odorants tested at  $10^{-2}$  dilutions (in paraffin oil). 2-ethylphenol and o-cresol were included in the original odorant panel (Figure 1) and are shown here for activity comparisons. N=3 to 6. Error bars are standard deviations. **B.** AqOr9 responses for odorants tested at  $10^{-4}$  dilutions. N=6 to 9, except for 4-methylguaiacol N=3.



**Figure 5: AqOr9 odorant dilution series.** Each point represents odorant induced response of a different ab3A neuron. Bars represent the average response.

**Chapter 4:**  
**Manipulation of odorant receptors in *An. stephensi***

## Abstract

Utilizing data on mosquito odorant receptor function, we developed a proof-of-concept method to alter host choice in *An. stephensi*. *An. stephensi* odorant receptor, Or8, is sensitive to human odorants. A second odorant receptor from *An. quadriannulatus* interacts most strongly with aromatics, in particular guaiacols. We created a construct to replace expression of AsOr8 in the *An. stephensi* capitate peg b neuron with AqOr9, using Cas9 induced double-strand break followed by homologous recombination. The odorant receptor replacement trait could be coupled to a gene drive, potentially preventing human feeding and disrupting disease transmission. We were ultimately unable to generate a transgenic mutant *An. stephensi* with Cas9 due to the challenging technical nature of the problem. We discuss advances in Cas9 gene editing in mosquitoes in the concluding sections of chapter. These advances may make implementation of gene drives to alter mosquito host choice more feasible in the near future.

## 1. Introduction

Differences in feeding behaviors have significant consequences for the vectorial capacity of *Anopheles* mosquitoes. Mosquitoes in the genus *Anopheles* vector malaria, which resulted in over 600,000 deaths in 2019 (WHO World malaria report 2021). Yet many species of *Anopheles*, including *An. quadriannulatus*, are competent for malaria but are not significant vectors (Takken & Verhulst, 2012). A defining characteristic of the most important vectors of malaria, which includes the significant urban vector *An. stephensi*, is a proclivity for feeding on human blood. Among multiple sensory cues which influence mosquito behaviors, olfaction is important to host preference (DeGennaro et al., 2013; McBride et al., 2016).

We previously characterized mosquito odorant receptors (ORs) from anthropophilic (See Chapter 2, *An. stephensi*) and zoophilic species (See Chapter 3, *An. quadriannulatus*) of mosquito. Using information from mouthpart transcriptome profiles, we identified and

characterized *An. stephensi* ORs which could play a role in the human host choice behavior of this mosquito. *An. stephensi* Or8 is activated by several human odorants, especially the abundantly human-emitted odorants sulcatone and 1-octen-3-ol, at low concentrations (Speth et al., 2021). Other odorant receptors characterized were unresponsive to human odorants. In contrast, an odorant receptor from *An. quadriannulatus*, AqOr9, has a very different activity profile, and was responsive to mostly aromatic molecules, most notably guaiacols (Chapter 3). An *An. stephensi* neuron in the maxillary palp capitata peg sensilla responds to 1-octen-3-ol and has an odorant induced activity profile which closely matches *D. melanogaster* ab3A ORNs transgenically expressing AsOr8 (Speth et al., 2021). This neuron is arranged adjacent to the CO<sub>2</sub> sensory neuron, and this activity pairing is broadly conserved amongst diverse mosquito species, including *Aedes aegypti*, *Toxorhynchites amboinensis*, and *An. gambiae* (Lu et al., 2007; Bohbot et al., 2013; Dekel et al., 2016).

An Orco mutation disabling all ORs has previously been shown to ablate human host preference of *Ae. aegypti* mosquitoes demonstrating the importance of Ors in discriminating host odors (DeGennaro et al., 2013). The objective of the current study was to test whether manipulation of OR genes can be used to alter host choice. Specifically, this study aims to address whether the manipulation of a single olfactory receptor neuron through substitution of the native OR or deletion or knockdown of the endogenously expressed OR can alter mosquito behavior. For this study, we targeted the human odorant sensing *An. stephensi* Or8 and attempted to replace it with *An. quadriannulatus* Or9. While we were unable to achieve this aim in the current study, this work provides a framework for future efforts to manipulate mosquito host seeking behavior. This report concludes with a summary of recent developments in gene editing of insects and describes approaches to improve future research in this area.

## 2. Methods

## Cloning

To assemble a transgenic construct to replace AsOr8 activity with AqOr9 activity upon homologous recombination (Figure 1), In-Fusion Cloning was used according to kit directions (Takara Biosciences, Mountain View, CA). Each fragment was PCR amplified with primers appending 18-25 bp 5' overhangs overlapping the adjacent fragments (Table 1). Genomic DNA extracted from *An. stephensi* was used as template for amplification of the homology arms (LHA and RHA), cDNA prepared from head RNA extracts from *An. quadriannulatus* was used for AqOr9 amplification, and 3x3P and EGFP products were amplified using a pBAC-EGFP plasmid template. Fragments were assembled into a EcoRI digested pUC19 vector, using multiple-insert cloning. 5' homology arms match the AsOr8 5' UTR and range in length from 500-700 bp. EcoRI sites were added back to the 5' end of the construct to allow for linearization with restriction enzyme digestion.

Prior to multiple-insert cloning, PCR products were cleaned using Nucleospin columns (Macherey-Nagel). Fragments were added at the following molar ratios to linearized pUC19 vector to the Nucleospin column: H01, H02, AqOr9 and EGFP at 2:1 molar ratios; 3x3p at a 4:1 molar ratio. The respective masses of each fragment added to the clean-up reaction were 60 ng H01, 81 ng H02, 58 ng GFP, 49 ng 3x3p, 100 ng AqOr9 and 103 ng linearized pUC19.

Fragments were cloned into a transgenic vector construct using either In-Fusion multiple-insert cloning with Takara EcoDry (Takara Bio) or multiple insert cloning using NEBuilder HiFi DNA Assembly (New England Biolabs), following manufacturer's protocols. 2.5 ul of the assembled product from multiple-insert cloning assembly reactions was added to NEB competent cells. The cells were transformed following the manufacturer's instructions. Cells were incubated for one hour on agar supplemented with 100 ug/ul, and then flipped and incubated

overnight. See Chapter 3 methods for cell growth and plasmid extraction. Plasmids were screened for the transgene insert using PCR and Sanger sequencing.

### sgRNAs

sgRNAs targeting both the AsOr8 5' UTR (for inserting transgene via homologous recombination) and the Or8 open reading frame (for gene KO) were selected using ChopChop (Table 2). sgRNA templates were prepared through PCR amplification of forward primers which consisted of the target sgRNA sequence, appended with a 5' T7 promoter and a 3' 20 bp overlap with the tracrRNA. The reverse primer in each case was the reverse complement of the tracrRNA. sgRNAs were amplified using T7 transcription, after PCR amplification of sgRNA dsDNA templates (see Table 3).

### Microinjections

\_\_\_\_\_ Embryo microinjections were performed by adaptation of a protocol described in Li et al., (2018), described here as follows:

Each embryo injection mix consisted of 100 ng/μl NLS-tagged Cas9 protein (PNA Biosciences), 100 ng/μl of each respective repair template, and 100 ng/μl of each respective sgRNA. Separate injection mixes were prepared for each repair template with the respective sgRNA.

Female *An. stephensi* were given a 30 min period for forced egg laying. 7-20 female mosquitoes blood fed 4-10 days previously were used for each round of forced egg laying. Female *An. stephensi* lay the most eggs 5-7 days after feeding, with numbers decreasing on subsequent days. Immediately after the forced egg laying period, 20-50 embryos were aligned on wetted Whatman filter paper. The embryos were aligned against glass coverslips. The alignment was performed within 20-30 min periods. Longer alignment periods allowed the endochorion to start hardening. Microinjections to embryos with hardened endochorion result in greater damage and decreased survival post-injection.

Immediately after the alignment, the filter paper was quickly dried, and embryos were fixed to tachoderm tape on prepared slide. After the transfer to tachoderm, the embryos were immersed in hydrocarbon oil. *An. stephensi* embryos are very sensitive to desiccation, and each of these steps were performed quickly to prevent embryos from desiccating.

Embryos immersed in oil were nanoinjected on an injection platform. Injections were performed at the posterior end of embryos, near to the developing germline cells. Throughout injections the pressure, transfer, and injection time parameters on the injector were varied according to droplet size visible in the oil immersion. Needles were opened by rubbing on either filaments or the end of embryos and increasing pressure. Needles were replaced if they were either unable to be opened by rubbing, or if the tip was too blunt.

After the injections, embryos unable to be successfully injected were deliberately destroyed. The successfully injected embryos were gently washed in deionized water and immersed in a shallow layer of deionized water in large petri dishes. The injected embryos were carefully monitored, and supplied with food once larvae began to emerge. After allowing a few days after the first few larvae emerge, slides were transferred to larger trays used for mosquito rearing. The larvae were fed only as necessary to allow for development.

The eyes of both 4<sup>th</sup> instar larvae and adults were screened for green fluorescence using a fluorescence microscope. Adult mosquitoes which survived injections were allowed to mate, and the offspring were screened for green fluorescence during the 4<sup>th</sup> instar larval stage.

#### RNAi knockdown of AsOr8

Primers were designed to amplify ~500 bp template regions of AsOr8 for the generation of dsRNA as described in Nuss et al. (2018). T7 Megascript (Invitrogen) was used to manufacture the dsRNA, following manufacturer's instructions. The dsRNA was injected into 4 day old, cold-anesthetized *An. stephensi* adult females. Mosquitoes were allowed to recover and RNA was extracted 3 or 5 days later, and was used to generate cDNA for use in qRT-PCR

(Deepak et al., 2007). In addition, pupae were treated with dsRNA according to the protocol outlined in Arshad et al., (2021). Pupae were allowed to emerge as adults and RNA was collected at days 2,4, or 6 after injection. GFP dsRNA was used as a control in both experiments.

### 3. Results

In total, out of 3,006 *An. stephensi* eggs injected with AsOr8 sgRNAs and AqOr9 repair construct, survival was 5% and no GFP expression was detected in F1 larvae or adults. F1 adults were allowed to mate, blood feed, and resulting F2 progeny were also screened, but again no insects expressing GFP were detected. Microinjection variables impacting *An. stephensi* embryo survival were also tested (Table 3). Even without injecting, the alignment and transfer steps caused significant deaths amongst *An. stephensi* embryos. These results suggest that either the transfer or short period of desiccation is lethal, or the double-sided tape may be toxic to the embryos.

Adult mosquitoes injected with dsRNA or pupae exposed to dsRNA showed no significant knockdown of AsOR8 transcripts in comparison to GFP RNAi controls (Fig 3).

### 4. Discussion

The attraction of mosquitoes to host odors has long been known, yet only recently have the finer details of host choice been explored on a molecular level. The role of ORs in host choice has been demonstrated with the heavy-handed knockout of Orco, rendering all Ors inoperative (Degennaro et al., 2013), but has also been suggested on a more nuanced scale with the discovery of Or2 mutants in generalist (forest) and anthropophilic (urban) strains of *Ae. aegypti* (McBride et al., 2014). In addition, several mosquito Ors have been characterized in cell expression systems or in the *Drosophila* empty neuron system (Carey et al., 2010; Wang et al., 2010). These studies reveal that several mosquito Ors are sensitive to odors disproportionately abundant in the human

volatile spectrum (Carey et al., 2010; Wang et al., 2010). With this information, we proposed that host choice of vector mosquitoes might be altered by the knockout or substitution of Ors sensitive to predominantly human-emitted odors. In particular, the most human sensitive Ors could be replaced with Ors sensitive to odors emitted in greater abundance by other animals in the environment. If such a strategy is successful, this trait could be coupled to a gene-drive mechanism and spread into the mosquito population (Hammond et al 2015; Gantz et al 2015). A population-wide disruption in human feeding behavior could break human-mosquito disease transmission cycles. This strategy contrasts with current mosquito control strategies such as the use of chemical insecticides that incur high selection pressures on mosquito populations to develop insecticide resistance. This approach could also limit the environmental impacts of chemical insecticides, which are not species-specific. This strategy would also be resilient in the face of disruption of chemical control measures, whether due to natural disasters, lack of funding, or human conflicts. Further, a gene-drive spread human avoiding trait still allows mosquitoes to blood feed on other animals in the environment and reduces selection pressure for resistance traits. This trait could be coupled to other traits such as refractoriness to malaria infection in the mosquito itself, setting up further barriers to disease transmission.

## **5. Future directions**

Despite the promise and purported ease of use of CRISPR-Cas9, technical hurdles remain in the applicability and repeatability of this technology, particularly for non-model organisms such as *An. stephensi*. To date, successful transformations in mosquitoes require high numbers of embryo injections and incur a high mortality rate (Gantz et al., 2015; ). However, technological improvements in gene editing with Cas9 can improve gene editing efficiency in mosquitoes, and could be applied in future studies to manipulate olfactory driven behaviors in mosquitoes. Guide RNA stability and efficiency can be increased through the incorporation of chemically modified

nucleotides (Rahdar et al., 2015). Sequence modifications to the tracrRNA can also greatly increase the editing efficiency of Cas9 in mosquitoes and fruit flies (Feng et al., 2021). A modified tracrRNA which incorporates a 5 bp hairpin extension, increased editing efficiency 5 - fold (Feng et al., 2021). Further, the greater availability of microinjection services would render the high burden of microinjection skill learning less of a barrier for the availability of transgenic mosquitoes to a wider circle of research laboratories. Such services exist for *D. melanogaster* and may soon be more widely available, but for mosquitoes only a few specialized labs are currently able to routinely generate transgenic insects.

An editing strategy not applied in this study is the injection of Cas9 and sgRNAs expression vectors into the mosquito embryo. Distinct functions can be engineered into expression vectors, which are not available using direct injection of Cas9 protein. *Vasa*-driven Cas9 constructs can be selectively expressed in the *Anopheles* germline (Papathanos et al., 2009; Gantz et al., 2015). A more efficient germline promoter, *nos*, was recently discovered. Other genes can be delivered in vectors to bias DNA repair mechanisms. For example, expression cassettes can be designed to express Ku70/80 knockdown guides to increase the ratio of homologous to non-homologous recombination (Gantz et al., 2015).

Embryo microinjections, which are especially lethal to *An. stephensi*, can be circumvented entirely with the use of a modified Cas9 protein dubbed 'ReMOT Control' (Macias et al., 2020). This transgenic Cas9 is tagged with a yolk protein fragment which allows deposition into the ovaries during early embryogenesis within adult female mosquitoes (Chaverra-Rodriguez et al., 2018). This method allows for mosquito lines to be edited by injecting adult females and subsequently screening progeny for target mutations, and is less time consuming and requires less expertise to execute than embryo microinjections (Chaverra-Rodriguez et al., 2018). However, the ReMOT method currently lacks the ability to effectively deliver repair template into the embryo. This is currently an impediment to generating Cas9-

mediated transgenic insertions with ReMOT. However, developments in ReMOT and other gene-editing advances could greatly increase the accessibility of genome editing in mosquitoes in the future.

During our attempts to make a transgenic, olfactory mutant *An. stephensi*, we encountered several difficulties stemming from embryo survivability and Cas9 editing efficiency. Improvements to editing efficiency through the use of modified guide RNAs and improved germline promoters promise to overcome many of the current challenges to generating transgenic insects. The use of ReMOT Control technology also substantially reduces the technical expertise and time demand requirements of utilizing CRISPR in insects. Future efforts to generate olfactory mutant mosquitoes could achieve greater successes by incorporating these developing gene editing technologies.

**Table 1: Primers used to PCR amplify products for In-Fusion cloning of the OR transgene substitution construct.** Gray highlights are shown to differentiate 5' overhangs with adjacent fragments.

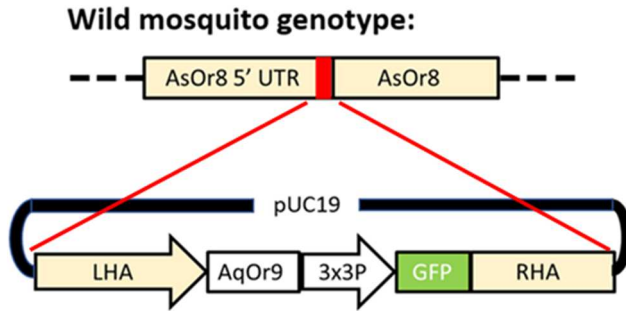
Primer name	Fragment	Sequence
Hol_AsOr8F_EcoRI	LHA	TGTA AACGACG GCCAGTGAATTCGGGGGGGAATAAGAAGTCTGC
5' pUC19_EcoRIR_Or8	LHA	CAGACTTCTTATTC CCCCCCGAATTCACTGGCCGTCGTTTTACAA
Hol_G1_Or8Rnew	LHA	CGGAAGAAGAGCCTAACCATAGTTCAGCGAACATCCTC
AqOr9_G1_Or8Fnew	AqOr9	GAGGATGTTTCGCTGAACTATGGTTAGGCTCTTCTTCC
AqOr9_G_Or8_41Rnew	AqOr9	CGGGCGAGCTCGAATTACTAATCCGTCATCGATCTC
3X3P_G_Or8Rnew	3x3P	CTCCTCGCCCTTGCTCACCATGGTGGATCCCGGGCC
GFP_G_Or8Fnew	GFP	CCGCGGGCCCGGGATCCACCATGGTGAGCAAGGGCGAG
GFP_G1_Or8Rnew	GFP	CCAGAACCACAGACCTCCTAACTTGTACAGCTCGTCCATG
Ho2_G1_Or8Fnew	RHA	CATGGACGAGCTGTACAAGTTAGGAGGTCTGTCGGTTCTGG
Hol_G2_Or8Rnew	LHA	CGGAAGAAGAGCCTAACCATCATTGCATTGGTGAACCTCG
AqOr9_G2_Or8Fnew	AqOr9	CGAGTTCACCAATGCAATGATGGTTAGGCTCTTCTTCC
GFP_G2_Or8Rnew	GFP	GATTGAGCACTGATGAGTTCCCTAACTTGTACAGCTCGTCCATG
Ho2_G2_Or8Fnew	RHA	CATGGACGAGCTGTACAAGTTAGGGAACCTCATCAGTGCTC
Hol_G3_Or8Rnew	LHA	CGGAAGAAGAGCCTAACCATAAATTGGAACGCGGACAAG
AqOr9_G3_Or8Fnew	AqOr9	CTTGTCCGCGTTCCAATTATGGTTAGGCTCTTCTTCC
GFP_G3_Or8Rnew	GFP	CTGGAGTGGTCGGTATTTAAAGGTAACCTTGTACAGCTCGTCCATG
3XP3_G_Or8_41Fnew	3x3P	GAGATCGATGACGGATTAGTAATTCGAGCTCGCCCCG
Ho2_G3_Or8Fnew	RHA	CATGGACGAGCTGTACAAGTTACCTTTAAATACCGACCACTCCAG

**Table 2: sgRNA target sites for the Or8 5' UTR.** The gray highlights indicate N to G substitutions implemented in the sgRNA design.

sgRNA name	Length (bp)	Target sequence	PAM	Off-targets
AsOr8_5'_180857	20	GGCAA <b>ACT</b> CTACCGATCAGC	TGG	none
AsOr8_5'_180740	19	GGTCGGTATTTAAAGGAAT	TGG	none
AsOr8_5'_180666	20	GGG <b>TT</b> CACCAATGCAATGGG	GGG	none
AsOr8_5'_180855	20	GGCTGATCGGTAGAGTTTGC	TGG	1:3
AsOr8_5'_180602	19	GGATG <b>TT</b> CGCTGAACTTGG	AGG	none

**Table 3: Survival test for different steps of embryo microinjection handling.**

Test type	Replicate	# Hatched	# Eggs	% Survival
Hatch test (no handling)	1	72	115	63%
	2	73	195	37%
	3	48	103	47%
Hatch test, alignment, no slide transfer	1	37	139	27%
	2	62	165	38%
	3	16	137	12%
Hatch test, alignment and transfer to slide	1	6	85	7.0%
	2	3	48	6.3%
	3	7	128	5.5%

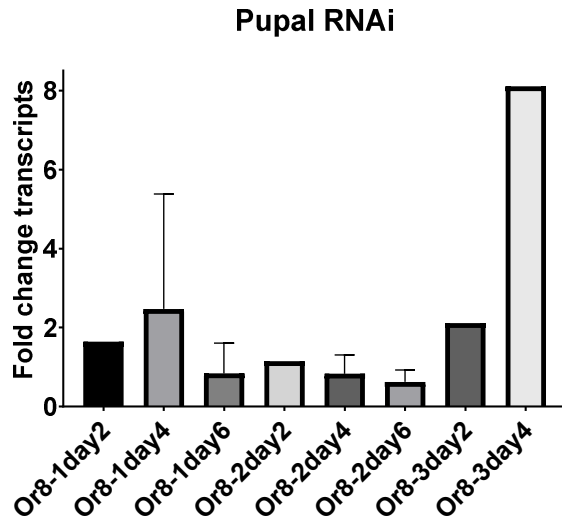


**Figure 1: Schema of the transgenic construct for replacing *AsOr8* with *AqOr9*, in the endogenous, *AsOr8* expressing *An. stephensi* ORNs. Red highlights the region targeted for Cas9 cleavage directly upstream of the *AsOr8* start codon. LFA = left homology arm, RHA = right homology arm.**

CCCTC<sup>A</sup>ACATGAGTGTCCAGTCCAGGGTGTGCGCTCGTGCACTGCCCCAGCTCTGGACGACTACTGGTAAGGG  
 GATAAAAAGGAAGTACTCGAACCCGGACCCGTTTGGTTGCATTCTGGAGTGCCTCAAGGCATCTCATT  
 ATAGACTTCAAGCTCTGCTCTGCTGTCCCATATCGGACGCCACACCTAACATGAAAAATATCGATAAAAAAT  
 ACCGACGTTAGGTCCCAGGAATATGGCATCGTACCTAAAAACGGTTCAAGTGTTTATGTATCTTAAACAAG  
 CTCACACAATGTACCACCAGGAGTAGTATGTCCCGTAAAAGGGGGATGTTAAAAATAGCCTAAGAATCTC  
 AGCCTCATTAGTCACGTGTGTGTGGTTCTCGGGGTGGATTTTTCCAATAGACCGTTAAACGTATCGATCGA  
 TTGACTTTATGTTGA<sup>GGATGTTGCTGAACTTGGAGG</sup>TCTGTGCGTTCTGGTTTTGGGCGCAGAGTTCCGA  
 CTCTTGGTCTACTCAGGTCTACTCCACAAAGCTGTATTGATCCTTCCATCCATAATCTATGTATTCAC<sup>GA</sup>  
<sup>GTTACCAATGCAATGGGCGG</sup>GAACTCATCAGTGCTCAATCAGTGCATATGTTATCAACGCTTGTCCGCGT  
<sup>TCCAATTCCTTTAAATACCGACC</sup>ACTCCAGCACAACCCTGCACAACCTCAAACGATTACTATAAGT<sup>T</sup>GCT  
 TTATAGAGGCTTTGATTCTGGCACACACAGAAG<sup>a</sup>aaaaaa<sup>ATG</sup>CCAC<sup>CCAGCAAACTCTACCGATCAGCTGG</sup>  
 TGCAATTCGAATC

- Or8 transcription start site
- PAM
- Insertion
- SNP
- Suggested N → G substitution position for gRNA design

**Figure 2: 5' UTR of AsOr8 showing sgRNAs and target cleavage sites.** Start codon is highlighted in red. Cas9 most frequently cleaves 3 or 4 base pairs 5' to the PAM.



**Figure 3: Knockdown efficiency of RNAi by pupal soaking.** Fold change transcripts normalized against GFP control. Or8-1day4: N=4; Or8-1day6: N=3; Or8-2day4: N=4; Or8-2day6: N=3.

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