Understanding the Role of Odorant Receptors in *Aedes aegypti* Larvae

by

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ABSTRACT

Worldwide, mosquitoes are of great medical and economic importance due to their profound negative impact on humans. Mosquitoes act as both nuisance pests and disease vectors that account for millions of cases of illness and death annually. They rely on complex olfactory systems for many of their fundamental processes such as finding mates, identifying egg-laying sites, and directly linked to their disease vectoring capabilities, locating blood-meals. Their olfactory systems rely on three major categories of receptors: odorant receptors, ionotropic receptors, and gustatory receptors. These receptors, along with sensors of various environmental cues such as CO_2, heat, and humidity, contribute to the mosquito's chemosensory responses. The yellow fever mosquito, *Aedes aegypti*, is responsible for the transmission of key diseases such as dengue virus, yellow fever virus, chikungunya virus, and Zika virus. Because of its profound impacts on human health, there is growing interest in exploring new ways to disrupt *Ae. aegypti's* olfactory-based behaviours as a means of reducing disease transmission. While there are numerous studies focused on adult mosquito chemoreception, little is known about these processes in mosquito larvae. Here, a chemotaxis response assay was developed to examine which naturally occurring chemicals would attract *Ae. aegypti* larvae. Mosquito larvae were subsequently fed *E. coli* bacteria expressing double stranded RNA targeting a larval specific odorant receptor, OR34. RNA interference knockdown of the gene's transcript was confirmed via qRT-PCR, and chemotaxis assays were used to identify putative odorant ligands to this receptor. This research describes a simple chemotaxis response assay that can be used to identify larval mosquito attractants and an effective way to identify odorant receptors' potential ligands. Using this information, it should be possible to screen for other ligands that may be used to disrupt mosquito olfaction, and thus develop novel larval mosquito control methods.

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LIST OF ABBREVIATIONS

Amp	Ampicillin
BLAST	basic local alignment search tool
CDS	coding sequence
CRIPSR	clustered regularly interspaced short palindromic repeats
DEET	N, N-Diethyl-meta-toluamide
dsRNA	double-stranded RNA
GR	gustatory receptor
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IR	ionotropic receptor
LB	lysogeny broth
mRNA	messenger RNA
OR	odorant receptor
ORCO	odorant receptor co-receptor
PCR	Polymerase chain reaction
PDB	Protein data base
qRT-PCR	quantitative reverse transcriptase PCR
RNAi	RNA interference
siRNA	short interfering RNA
Tet	tetracycline

1 INTRODUCTION

1.1. Insect olfaction

1.1.1 Overview and significance

Insects are our most important competitors for food and fiber and are vectors of some of our most serious diseases (Purcell and Almeida 2005; Belluco *et. al.* 2023). Most pest insect species have been and continue to be controlled using chemical pesticides. Continuous use of insecticides has raised two serious issues: 1) insects are rapidly developing resistance to many of these chemicals (reviewed in Stanczyk *et. al.* 2010) and 2) most insecticides have off-target, toxic effects on other species (Aktar *et. al.* 2009). Mosquitoes are among the many insects where these problems with insecticides have been frequently observed (Richard and Byrd 2020; Liu 2015). Even the best insecticide deployment strategies cannot fully eliminate mosquitoes, and hence another common approach to reducing the spread of mosquito borne diseases is through the use of mosquito repellents. The most effective mosquito repellents contain DEET, a compound that successfully interferes with the insect's olfactory-based systems to find a host, but unfortunately, prolonged use poses health risks to humans (Robbins and Cherniak 1986). For this reason, there is increasing interest to find some new alternative repellent strategies. Inhibiting mosquitoes' ability to smell will limit their ability to detect hosts and in turn limit disease transmission.

1.1.2 Overview of mosquito olfaction

Mosquitoes rely on their olfactory systems for many fundamental processes, including but not limited to, finding mates, locating egg-laying sites, and directly linked to their disease vectoring capabilities, detecting hosts to acquire blood-meals by the female mosquitoes (Konopka *et. al.* 2021). The olfactory system does not operate alone but rather works in

coordination with the mosquito's visual system while also integrating various other sensory cues such as heat, CO_2 and moisture (San Alberto *et. al.* 2022). Certain odors or olfactory-based cues can stimulate visual search behaviours in *Aedes* mosquitoes. For example, in the presence of CO_2, *Aedes aegypti* mosquitoes were subsequently found to exhibit a strong attraction to objects emitting colour wavelengths that humans perceive as cyan (496 nm), orange and red (>590 nm) (San Alberto *et. al.* 2022). This study thus illustrated the value of evaluating the whole organism rather than just its individual sensory systems to gain a more accurate description of how mosquitoes locate hosts. Despite this study into the complexities of mosquito host-finding behaviour, much of what we know about mosquito olfaction is nevertheless based on a reductionist's view of individual components of the insects' olfactory system.

1.1.3 Olfactory receptors

Mosquitoes have three types of olfactory receptors: odorant receptors (ORs), ionotropic receptors (IRs) and gustatory receptors (GRs). ORs are heteromeric, ion gated channel receptors that work in coordination with the highly conserved odorant receptor co-receptor (ORco). Their structure closely resembles that of G-protein coupled receptors (GPCR) with multiple intracellular, extracellular, and (typically seven) transmembrane domains (Kythreoti *et. al.* 2020). Specific ligands binding to the ORx-ORco complex triggers odorant-dependent behaviours in mosquitoes such as food-seeking and reproduction. Ionotropic receptors are also heteromeric ligand-gated transmembrane proteins that function with a co-receptor. However, unlike ORs with only one co-receptor, there are three IR co-receptors: IR8a, IR25a and IR76b (Raji *et. al.* 2019). In the vinegar fly, *Drosophila melanogaster*, IRs have been described as multimodal receptors responsible for detecting odorants, taste, humidity, and cooling sensations

(Wicher and Miazzi 2020). Gustatory receptors are the most diverse of the insect chemoreceptors. The family consists of receptors responsible for taste, pheromones, detection of CO_2, and other non-volatile chemicals (Kent *et. al.* 2007; Montell 2013). Both their structures and their functions are much more diverse than the ORs and IRs, but their structures often also resemble that of GPCRs (Konopka *et. al.* 2021). A key gustatory receptor for mosquitoes is Gr3, which is responsible for the detection of CO_2 (McMeniman *et. al.* 2014). With an estimated 131 ORs, 135 IRs, and 114 GRs identified in the *Aedes aegypti* genome, this mosquito clearly has a diversity of odorant detecting molecules, which coupled with other sensory modalities such as vision and heat detection, provides the insect with a varied arsenal of host-finding mechanisms, to ensure that females find the needed blood meals to nourish their eggs (Bohbot *et. al.* 2007; Matthews *et. al.* 2019; Kent *et. al.* 2007).

1.1.4 Overview of *Aedes spp.*

The *Aedes* genus of mosquitoes consists of over 950 species including *Aedes aegypti* (Yellow fever mosquito) and *Aedes albopictus* (Asian tiger mosquito). The life cycle of *Aedes* mosquitoes consists of four stages: egg, larva, pupa, and adult. The larvae live in water and mature through four instars (Rogers 2019). Days after eclosing, adult mosquitoes mate and females seek their first blood meal, which is required for their egg production. *Aedes aegypti* and *Aedes albopictus* mosquitoes play a key role in the transmission of various viral pathogens such as dengue virus, yellow fever virus, chikungunya virus, Zika virus, and dirofilariasis (a nematode parasite), among others (European Centre for Disease Prevention and Control 2022). In 2020 in the Americas, 2,326,115 cases of dengue, 103,002 cases of chikungunya and 22,923 cases of Zika were reported (Pan American Health Organization 2020). Worldwide, it is estimated there

are upwards of 390 million cases of dengue and thousands of deaths per year. The number of reported dengue cases has increased 8-fold over the past two decades (World Health Organization 2022). Severe dengue is one of the leading causes of hospitalization and death in some Asian and Latin American countries. *Aedes* mosquitoes also transmit yellow fever virus which causes roughly 200,000 cases of disease and 30,000 deaths per year (Centers for Disease Control and Prevention 2018). Although many mosquitoes act as vectors for numerous pathogens, the diseases that *Aedes* mosquitoes transmit have devastated humans for centuries, making the genus of key importance in our efforts to stop disease transmission (Powell 2018).

1.1.5 Host odorant detection in *Aedes aegypti*

Several *Aedes* species, including the primary dengue vector *Aedes aegypti*, are classified as anthropophilic mosquitoes, meaning they prefer to feed on human hosts rather than other animals (Raji *et. al.* 2019). Human body odor is composed of a blend of attractive volatile compounds created largely through the skin's microbiome, allowing for easy detection and identification by mosquitoes. Human odors such as ammonia, amines, carboxylic acids, ketones, sulfides and octenol have been observed to elicit electrophysiological and behavioural responses in *Aedes aegypti*. Using electroantennogram analyses in this species, where odorants are directed over isolated antennae connected to microelectrodes, IRs were observed to detect amines, aldehydes, ketones, and acids whereas ORs detect alcohols and esters. Therefore, IR and OR odor ligands do not appear to overlap (Raji *et. al.* 2019).

1.1.6 Differentially expressed olfactory receptors

All three categories of olfactory receptors are differentially expressed across species, sexes, developmental stages, and tissue types. Receptors can also be expressed differently depending on environmental conditions and external stimuli. Different mosquito species or different laboratory strains of the same species can also have distinct olfactory gene expression (Mitra *et. al.* 2021). For example, divergence of the expression and odor sensitivity of *OR4* between *Aedes* species has been linked to different degrees of attraction to humans (Athrey *et. al.* 2020). Chemosensory olfactory genes can also be female or male biased. This is important to consider as they may modulate important sex-specific behaviours (Athrey *et. al.* 2020). Female mosquitoes are more attuned to scents involved in locating hosts and oviposition sites, whereas male mosquitoes are more attracted to plant volatile compounds as they feed only on flower nectars. Mosquitoes also express olfactory receptors differently depending on their life stage. As a mosquito matures through the larval, pupal, and adult stages, different olfactory receptors will be expressed at varying levels (Bohbot *et. al.* 2007). For example, in *Aedes aegypti*, adult mosquitoes express 80 odorant receptors, whereas their larval counterparts express only 23, 15 of which are larval specific (Wheelright *et. al.* 2021). Typically, olfactory gene expression will be enriched in olfactory tissues compared with the rest of the mosquito body. Some non-olfactory tissues, such as the legs, can have small quantities of olfactory receptors, although their role in odorant detection has not been well defined (Leal *et. al.* 2013; Wicher and Miazzi 2020). The primary olfactory sensory organs in mosquitoes are the antennae, the maxillary palps, and the labella on the proboscis (Bohbot *et. al.* 2007). These structures are all covered with sensilla containing olfactory sensory neurons that express different combinations of ORs, IRs and GRs (Konopka *et. al.* 2021). Expression can also change based on various environmental factors such as if a female

has recently blood-fed or laid eggs (Das De *et. al.* 2018; Rinker *et. al.* 2013; Ni *et. al.* 2022). The fundamental ability to regulate and differentially express olfactory receptors is crucial to a mosquito's activities.

1.2 Applications in Insect Control

1.2.1 Overview and significance of insect control applications

Due to the importance of mosquitoes in relation to the spread of diseases affecting humans and livestock, there are numerous mosquito control methods already in place (Kent *et. al.* 2007). Currently, the most widely used approaches aim to control the growth of the mosquito populations by targeting different life stages of the insects (Whyard *et. al.* 2015; United States Environmental Protection Agency n.d.; Dusfour and Chaney 2022). An effective mosquito control method is the reduction of mosquito larvae through removal of their aquatic habitats (e.g. by removing standing water near human dwellings) or employing the use of larvicides in small water bodies (American Mosquito Control Association 2020). Broad-spectrum insecticides have been used for decades to control adult mosquitoes, but growing concerns about the adverse effects of these chemicals on other species has prompted renewed interest in species-specific control methods. Chemical insecticides are not only hazardous to other species and to human health but increasing numbers of pesticide-resistant strains of disease-vectoring mosquitoes are emerging at a rapid rate (reviewed in Lutrat *et. al.* 2019). Therefore, traditional control techniques are not as effective as they once were, and new, safer alternatives are desperately needed. In recent years, scientists have begun to explore molecular mechanisms for controlling mosquito population growth such as RNA interference (RNAi), using transgenes, and CRISPR (clustered regularly interspaced short palindromic repeats)-mediated gene drive.

1.2.2 Insect repellents

Insect repellents function through a variety of different mechanisms. For example, some repellents, like citronella, lemongrass oil, and eugenol, work by masking host odors or by confusing and overwhelming the sensory system (Afify *et. al*. 2019). The most popular and effective repellents contain DEET. The mechanism by which DEET works to repel insects is not yet fully understood. In *Drosophila melanogaster* and *Aedes aegypti*, DEET activates all OR-ORco complexes and likely functions by interacting with ORco directly (DeGennaro 2015). However, in the presence of CO_2, the ORco mutants were still able to host-seek. DEET does not mask detection of host odors but is thought to interact with the insect's ORs to alter their olfactory sensitivity. Other theories propose that DEET is a noxious odor for insects. The other olfactory receptors: IRs and GRs also play a role. For example, in *Drosophila*, Ir40a activates an aversive neural circuit in insects and GRs perceive DEET as bitter as a way for the insect to avoid consuming DEET containing food (DeGennaro 2015). Therefore, the molecular mechanism of DEET is complex due to the built-in redundancy in an insect's sensory system and interfering with one of these pathways alone will not be sufficient to successfully repel the insect.

1.2.3 RNA interference and current progress of RNAi against mosquitoes

RNAi is a sequence-specific post-transcriptional gene silencing antiviral immune response in eukaryotes (Vogel *et. al.* 2019). The process uses short interfering RNAs (siRNAs) as templates to guide the destruction of complementary transcripts. Within this pathway, an RNase III endonuclease, Dicer, cleaves double-stranded RNA (dsRNA) molecules into 21-24 base pair siRNA sequences. Following this processing, the RNA-induced silencing complex (RISC;

including Argonaute proteins) binds to the newly formed siRNAs. The siRNAs are unwound, and a single strand is retained by RISC to target the complementary messenger RNA (mRNA) transcripts for degradation. This results in post-transcriptional gene silencing (Alberts *et. al.* 2014). RNAi can be triggered artificially by introducing gene-specific dsRNA molecules. Delivery of these molecules can be done via micro-injections, soaking, feeding, microbial delivery systems or nanoparticles at different life stages of the target organism (Munawar *et. al.* 2020). A key advantage to RNAi is its sequence specificity which allows for accurately targeting transcripts within a pest species while having no adverse effects on other insect species (Whyard *et. al.* 2009; Baum *et. al.* 2007). However, RNAi can still pose some challenges, as some dsRNAs may have unpredicted shared sequence identities with non-target species, and hence would still require empirical testing to ensure they are safe to apply in the field.

In insects, two different dsRNA uptake pathways have been described: systemic RNA interference defective (SID) mediated uptake and the endocytic uptake pathway (Joga *et. al.* 2016). The SID-mediated pathway was initially described in *Caenorhabditis elegans* where the *Sid-2* gene encodes an intestinal membrane protein that imports dsRNA through endocytosis. Then the SID-1 transmembrane channel protein passively exports the siRNAs to other cells. *Sid-1* genes are present in many, but not all insect species, but to date, no *Sid-2* genes have been described in insects (Joga *et. al.* 2016). These SID-1 like channel proteins have been shown to be involved in dsRNA uptake in some insect species such as *Leptinotarsa decemlineata* (Colorado potato beetle) and *Diabrotica virgifera virgifera* (western corn rootworm) (Xu *et. al.* 2013; Cappelle *et.al.* 2016; Tomoyasu *et. al.* 2008; Whyard *et. al.* 2020). In some other insects, SID-like proteins have been identified, but knockdown of their genes did not impair dsRNA uptake,

which suggests that other mechanisms of uptake are used. For example, in *Bombyx mori* (silk moth) and *Tribolium castaneum* (red flour beetle) reduced expression of SID-like proteins had no impact on RNAi (Cappelle *et. al.* 2016; Tomoyasu *et. al.* 2008). In *Drosophila*, which like other dipteran insects (e.g. flies, mosquitoes), lack SID or SID-like genes, dsRNA uptake occurs exclusively through an endocytic pathway where dsRNA is taken up through receptor-mediated endocytosis (Ulvila *et. al.* 2006). Finally, Cappelle *et. al.* in 2016 found that both SID-1 like channel proteins and receptor-mediated endocytosis were involved with dsRNA uptake in coleopteran insects. Therefore, in insects, the role of each pathway in the uptake of dsRNAs differs depending on the species and can involve one or both described pathways (Joga *et. al.* 2016).

In mosquitoes, RNAi has been used to target genes that will impair the mosquito's ability to locate hosts, reproduce, develop, and survive, among others. A study conducted by Pridgeon *et. al.* in 2008 targeted an apoptosis inhibitor protein in *Aedes aegypti* which resulted in death in female mosquitoes. Lopez *et. al.* (2019) targeted *Aedes aegypti* chitin synthases which are required for larval development. This resulted in a significant reduction in the viability of the larvae, and the mosquitoes that did survive exhibited altered morphologies and lower chitin content. In addition to these insecticidal applications, RNAi has also been used for sterile insect technique (SIT), a process through which sterile males are released in the environment to compete with wild type males to mate with females, thereby reducing the number of progeny produced (Whyard *et. al.* 2015).

RNAi has also been used to target genes involved with olfaction. Pelletier *et. al.* (2010) targeted a gene encoding an odorant binding protein (OBP1) that binds the oviposition pheromone in *Culex quinquefasciatus* and observed that knockdown of OBP1 results in a significant reduction in mosquitoes' responses to oviposition attractants. Similarly, RNAi-mediated knockdown of the same gene in *Anopheles gambiae* mosquitoes reduced the insects' electrophysiological responses to the attractive compounds (Biessmann *et. al.*, 2010). Zhu *et. al.* (2013) knocked down OR37 and OR99, receptors that are narrowly tuned to specific phenolic compounds that elicit oviposition behaviour in *Culex quinquefasciatus*. Once treated with the dsRNA, the mosquitoes were unable to distinguish between the compounds and the water controls. Other studies have used RNAi to examine the role of GRs in olfaction. Erdelyan *et. al.* in 2011 assessed the *Gr1*, *Gr2* and *Gr3* genes in *Aedes aegypti* and found that knockdown of *Gr2* had no role in reception of CO_2 but knockdown of either *Gr1* or *Gr3* resulted in loss of CO_2 sensitivity. Overall, RNAi has many broad applications and can be an effective strategy for reducing the expression of select genes by creating loss-of-function phenotypes.

1.2.4 Genetically-modified mosquitoes using transposon-mediated transformation

Transposons naturally occur in the genome and are defined as mobile, repetitive segments of DNA that move from one location to another (Muñoz-López and García-Pérez 2010). Since the first use of P-element transposons to genetically modify *Drosophila melanogaster* in 1982 (Rubin and Spradling 1982), various transposon-mediated technologies have been developed to introduce novel transgenes in a range of insects (reviewed in [insert a review]), including mosquitoes. Using the piggyBac transposon, Alphey *et al.* (2011) engineered a transgenic strain of *Aedes aegypti* to have a repressible female-specific flightless phenotype,

for use in SIT-based population control. The OX360C transgenic strain contains tetracycline-repressible genes that produce a penetrant, dominant, late-acting lethal phenotype in female mosquitoes due to their inability to fly. This technology has been tested in field settings in the Cayman Islands. This location was selected for field trials due to its large native population of *Aedes aegypti* mosquitoes, the absence of other closely related mosquito species, its geographic isolation from other regions – limiting migration of mosquitoes to and from the islands, and its emerging prevalence of insecticide resistance (Harris *et. al.* 2011). By releasing sufficient numbers of genetically engineered males carrying a dominant lethal gene, the native mosquito population can be effectively reduced within months (Harris *et. al.* 2012). Their results demonstrate no reduction in fitness of the genetically engineered males and no differences in mating between wild-type mosquitoes. Their findings suggest that mass releasing genetically modified males is a viable method to control the spread of dengue by suppressing the *Aedes aegypti* population. The integration of this method along with the use of larvicides and removal of breeding sites would reduce the number of sterile male mosquitoes required to be released for effective population suppression. Overall, transposon-based genetic transformation is feasible in mosquitoes but because transposons randomly insert into the genome, the method requires screening many individuals to identify those that are stable and have limited impacts on the host. Due to these uncertainties with transposons, CRISPR-mediated genetic modification is seen as a better alternative.

1.2.5 CRISPR and current progress of CRISPR-based methods of mosquito control

The CRISPR-Cas system is a gene editing tool that harnesses a natural adaptive immune system found in bacteria (Henle 2019). The system provides a record of past infections by

expressing CRISPR RNAs (small RNAs that target the invasive nucleic acids). Repeats ranging in length from 23-50 nucleotides are separated by spacers of the matching sequences to the invasive DNA which identifies the CRISPR interference targets. Cas genes (CRISPR-associated genes) follow the repeat-spacers and encode the proteins necessary for CRISPR activity. Bacteria do not cut their own CRISPR DNA because the crRNA is complementary to both the spacer sequence and the repeat sequence flanking that spacer. The CRISPR-Cas system consists of three phases: acquisition of new spacers into the CRISPR arrays, expression, and processing of CRISPR-RNAs (crRNAs) and CRISPR interference.

Since its discovery, the CRISPR-Cas system has been adapted as a tool for genomic research, given its ability to cut DNA at specifically targeted locations. The most common CRISPR system for gene editing purposes is the type II, Cas9 complex. CRISPR-Cas9 can be used as a "cut-and-paste" method to modify genomes. It can edit the genome by removing genes, silencing/activating genes or introducing new genes (Mayo Clinic 2018). The system is composed of two important parts: the Cas9 protein and the guide RNA (sgRNA). The guide RNA recognizes the target DNA sequence through 20 nucleotide base-pairing interactions and directs Cas9 to the gene (Henle 2019). The Cas9 nuclease interacts with the protospacer-adjacent motif (PAM) of its DNA target through its PAM-interacting domain. Cas9 uses its nuclease domains to cleave the double-stranded DNA, creating a double-stranded break (Nature Video 2017). Gene editing can either be performed in somatic cells, where the mutation is only present in the tissue of interest, or in germline cells, where the mutation would be passed on to future generations. One important limitation when using CRISPR-Cas9 as genetic therapy is that it can

create off-target effects by generating mutations at random sites in the genome. It doesn't always only make the intended changes and so it is difficult to predict the long-term outcomes.

In mosquitoes, CRISPR has been used as an alternative to RNAi to target many of the same processes in an attempt to control mosquito populations. For example, Li *et. al.* in 2021 used CRISPR to apply SIT by disrupting *Aedes aegypti* genes required for male fertility and female flight resulting in successful population suppression. CRISPR-Cas9 has also been used in a gene drive system where the ability of *Anopheles gambiae* mosquitoes to transmit malaria is impaired. Gene drive refers to the phenomenon where a certain genetic element has a much higher probability of being inherited by offspring. This was explored in a 2016 study by Hammond *et. al.* where three genes involved with female fertility were disrupted by CRISPR-Cas9 gene drive constructs. They observed a strong gene drive resulting in transmission to progeny upwards of 91% for all three targeted loci, indicating that development of gene drives could suppress mosquito populations enough to impair transmission of malaria.

CRISPR has also been used to target a few olfactory target genes in mosquitoes. A recent study done by Raji *et. al.* in 2019 examined the importance of *Aedes aegypti* IRs in detecting human hosts. They examined IR8a, one of the IR co-receptors, which is responsible for detection of acidic volatile compounds such as lactic acid, a compound often enriched in human sweat. Using CRISPR-Cas9, the IR8a co-receptor was disrupted by generating null mutations in the gene. They found that the *IR8a* mutants had reduced attraction to human odorants. They also found that the loss of both *Ir8a* and *Gr3* causes a host-seeking defect similar to that caused by the loss of *Gr3* alone. This suggests that *Gr3* may be necessary for *Ir8a* function. Previous

studies have shown that the functional ablation of ORs or Gr3 is not enough to inhibit host seeking behaviour. This suggests that IRs do play a significant role in host detection. Therefore, CRISPR can be used to explore how the different olfactory receptors interact with one another and the role that they play in mosquito olfaction. Overall, gene knockdown and knockout strategies like RNAi and CRISPR can be highly effective mosquito control strategies.

1.2.6 Limitations and challenges in using transgenic insect control methods

The use of transgenic approaches in mosquito control will need to overcome numerous barriers. First, developing homozygous transgenic lines is expensive and requires a lot of time and effort (Strobl *et. al*. 2018). Most regions have strict regulatory restrictions that must be followed (Centers for Disease Control and Prevention 2022; Meghani 2022). For example, in the United States, prior to the release of genetically modified mosquitoes, the Food and Drug Administration (FDA) evaluates the ethical and environmental risks. As well, the use of genetically modified mosquitoes requires an experimental use permit granted by the Environmental Protection Agency. State and local authorities must also provide their approval. Lastly, the public has many negative perceptions about the use of transgenics and will need to be persuaded on its safety and efficacy (Wunderlich and Gatto 2015; Famakinde 2020; Resnik 2015). For example, members in a Florida community created and signed petitions to prevent the release of genetically modified mosquitoes into their environment (Resnik 2015). Others raise concerns that genetic modifications may not work as intended and could actually increase the rates of diseases transmitted by mosquitoes in the populations. Therefore, the release of genetically modified mosquitoes raises numerous social, ethical, and public health issues. Hence, non-transgenic technologies, such as RNAi, may have a continued role in mosquito control.

While many researchers considering RNAi for mosquito control are focused on insecticidal applications, there has been limited attention in the use of RNAi tools to interfere with insect olfaction.

1.3 Research objectives

Aedes aegypti mosquitoes rely on their olfactory systems for many of their fundamental processes, including finding blood meals. Due to their relevance to human health, disrupting this system is an important area of research as it could be a key component in limiting their negative impacts on society. Although there have been numerous studies on mosquito olfaction in adults, little is known about olfaction in larval systems. However, disrupting larval olfaction to stop the growth and development of larvae before they become blood feeding adults would be a novel approach to mosquito control that could complement more conventional insecticidal methods.

1.3.1 OBJECTIVE 1: Develop an assay to identify compounds that are attractive to *Aedes aegypti* larvae.

Using a novel chemotaxis assay, we can evaluate to which types of chemicals larvae are attracted. By understanding what types of chemicals attract mosquito larvae, we can develop larval traps that are specific to *Aedes aegypti* mosquito larvae. In this study, a quick, relatively cheap assay that can be used with any aquatic larval species to identify attractive compounds is described. It can also be used to assess the organism's sensitivity to various chemicals through a dose response assay.

1.3.2 OBJECTIVE 2: Identify the ligand for a key larval specific odorant receptor, OR34.

Identifying the ligand of larval specific odorant receptors may provide insight to help with developing novel larval control methods. Using the chemotaxis assay, the role of this OR in larval olfaction will be assessed through dsRNA-mediated transcript knockdown experiments. Knockdown experiments will also assess the impact of knocking down ORCO (herein referred to as OR7). Finally, both OR34 and OR7 will be simultaneously knocked down to assess whether this has a synergistic impact on their loss of the ability to detect previously attractive chemicals.

2 MATERIALS AND METHODS

2.1 Insects Rearing

Aedes aegypti adult mosquitoes were reared at 25°C at 50% humidity with a photoperiod of 16 hours of light and 8 hours of dark. Mosquitoes were fed a 10% sucrose solution. Females were provided fresh rat blood once a week to stimulate egg production. Eggs were laid on wet paper towels in water dishes and collected weekly. Eggs were hatched in plastic tubs filled with water with rabbit pellets supplied as nutrition. Larvae were reared at 28°C at 50% humidity until pupation, where they were collected and transferred into the adult mosquito cages.

2.2 Selecting Target OR Genes in *Aedes aegypti*

Ten ORs of interest were initially selected in *Aedes aegypti,* all of which shared close homologues, based on NCBI BLAST and Clustal Omega analyses, in *Aedes albopictus* (Supplemental Table 1). The amino acid similarities ranged from 98% similar (OR7) to 71% similar (OR87), with most falling between 85-90% identical. Half of these ten odorant receptors have known ligands and have been extensively studied (OR2, OR4, OR10, OR31, OR7), whereas the others have not been explored and have uncharacterized ligands (McBride *et. al.* 2014; Batra *et. al.* 2019, Liu *et. al.* 2021; Mitra *et. al.* 2021). By comparing the sequences of the odorant receptors between species we can observe any structural differences potentially impacting their function. Regrettably, the *Aedes albopictus* colony of mosquitoes died before any functional validation of ligands binding to their receptors could be conducted. Of the 10 *Aedes aegypti* odorant receptors selected, one odorant receptor, OR34 was unique, as it was identified as being predominantly expressed in early-stage larvae (Bohbot *et. al.* 2007), which was confirmed via qRT-PCR (See Results, Figure 1). As noted below, OR34 was only one of two

ORs that could be readily detected by qRT-PCR, and was therefore chosen for functional analyses, as it has an uncharacterized ligand.

2.3 OR34: Structure and Predicted Ligands

The 3D-structure of *Aedes aegypti* OR34 was predicted using Alphafold and drawn using ChimeraX and Robetta. A phylogenetic tree was constructed using MEGAX to determine the evolutionary relationship of OR34 in other mosquito and insect species. Using SwissDock, ligand-protein interactions were predicted using the best available homologue (Supplemental Figure 1). Through I-TASSER, PDB, and ConSurf, a protein identified as 7LICA was predicted to be the most structurally similar protein to *Aedes aegypti* OR34. The 7LICA protein PDB identifier corresponds to OR5 in *Machilis hrabei,* a jumping bristletail insect. The *Machilis hrabei* OR5 and *Aedes aegypti* OR34 proteins are ~85% structurally similar to one another and when their structures are overlayed using ChimeraX, it is difficult to distinguish them from one another (Supplemental Figure 2) Since OR34 was not characterized on PDB, the 7LICA protein was used for further structural and docking analyses (Supplemental Figure 1).

2.4 RNA Isolation and cDNA Synthesis

To assess the abundance of RNA of different ORs genes during development, insects of all developmental stages were collected and pooled together in groups of 10. To assess the abundance of RNA in different tissues of the insects, larvae and adults were chilled on ice and then using cleaned (ethanol and water rinsed) dissecting forceps, 10 heads, 10 thoraces and abdomens, and 10 tails of larvae were collected and pooled together. Three replicates of all samples (developmental stages and tissues) were collected. For most purposes, whole

mosquitoes for RNA analyses were collected at the 3rd instar larval stage. For developmental profile analyses, mosquitoes were collected at every stage of their life cycle. To assess which tissues OR34 was expressed in, larvae were dissected. Dissections included separating the larvae into three parts: the head, the thorax and the abdomen, and the tail. Dissections were performed by placing a whole larva in a well with 5 µL of lysis buffer. Tweezers were used to pull the head from the thorax and the thorax from the abdomen. Tweezers were cleaned with bleach, followed by ethanol, then water in between larvae. Respective tissues were pooled into groups of 10 and stored at -80°C. Tissue was placed in 300µL lysis buffer supplemented with 2% ß-mercaptoethanol and homogenized using a Bullet Blender (www.nextadvance.com). RNA was extracted using the GeneJET RNA purification kit (www.thermofisher.com). RNA samples were stored at -80°C. cDNA synthesis was performed using qScript cDNA SuperMix (www.quantabio.com). Synthesis of cDNA was done by adding the equivalent of 100 ng/µL of RNA to the reaction so that each qRT-PCR reaction will have relatively the same concentration of cDNA (and therefore RNA) added to the reaction. cDNA was diluted 1:4 for all PCR reactions unless otherwise specified. The absence of genomic contamination in the cDNA was confirmed via PCR amplification using primers from Table 1 to check for genomic contamination EconoTaq PLUS 2X Master Mix (www.biosearchtech.com) following the manufacturer's protocol. To visualize the samples, agarose gel electrophoresis was used to resolve the cDNA, with the cDNA stained with ethidium bromide and detected with a UV transilluminator.

Table 1. Primers used for this study.

Gene Name	Accession	Primer Type	Sequence 5'-3'
40S ribosomal protein S7	XM_001660119.2	qPCR	F: CCATTGAACACAAGGTCGACAC R: GTAGGGCTCCGGGAATTCGA
Aedes aegypti OR34	NM_001358623.1	qPCR	F: CACTTCGCACCAGTGAGTCG R: AGGCTGTGTTTAGAGTCGATGTG
		CDS	F: ATGGGTGAATTCGACGACTT R: CTTATTATTTTTGCTGTTGA
		dsRNA	F: TTTCAGTAGCCGGTCAGCAG R: TTGAGGTGCCGAGATGATCG
Aedes aegypti OR7	NM_001358471.1	qPCR	F: AAGCAGGAACTGATGGTCCGA R: ATGGCCGATACGAGGCGAA
		CDS	F: ATGAACGTCCAACCGACAAA R: TTTCAACTGCACCAACACCA

2.5 Quantitative Reverse Transcriptase PCR Analysis (qRT-PCR analysis)

qRT-PCR was used to assess relative levels of OR34 and OR7 gene expression in *A. aegypti* across the stages of development. An *Aedes aegypti* 40s ribosomal protein (s7), was used as an internal control and was amplified in order to normalize cDNA added to qRT-PCR reactions (Table 1). Gene specific qRT-PCR primers were designed to span exon-exon junctions (Table 1). Using the BioRad CFX Connect Real-Time PCR System, qRT-PCR was performed using SsoFast Evagreen Supermix (www.BioRad.com) according to the manufacturer's protocol. Melting curve analyses were performed to verify consistency of qRT-PCR products. Three technical triplicates of each reaction were performed to ensure precision. Transcript levels were quantified using the $2^{-\Delta\Delta CT}$ value method (Livak and Schmittgen 2001).

2.6 Chemical Preparation

Solid chemicals were dissolved in DMSO to produce concentrated stocks of 1M. Chemicals were subsequently diluted in molecular grade water to a concentration of 0.1 mM. Equal volumes of each individual chemical were then mixed with members of the same chemical groups (e.g. alcohols, aldehydes, ketones, etc.) (Table 3). A dose response chemotaxis assay (described below) was performed to assess the sensitivity of the larvae to the chemicals and 0.1mM was determined to be the best working concentration (See Results, Figure 8) (Huff and Pitts 2019).

2.7 Chemotaxis Assay

A chemotaxis assay to test larval attraction to various chemicals was developed. This assay used a plastic tray measuring 27 cm in length, divided into three equal lanes. Each lane was split

into three equal sections of 9 cm each. The three sections were respectively labelled “L” for left, “M” for middle, and “R” for right. On both the left- and right-hand sides of the tray, a 1 cm diameter filter paper disc was placed 2.5 cm from the edge of the tray. On the left-hand side, the filter paper disc was saturated with 5 µL of a chemical with a concentration of 0.1mM. On the right-hand side, the filter paper disc was the control. One minute after placing the filter papers in the water, 10 third instar larvae were gently placed in mid-point of the lane, between the filter papers. Initial screens tested groups of chemicals including alcohols, aldehydes, carboxylic acids, indoles, terpenes, and ketones. Chemical groups that showed more attraction relative to the negative controls were then tested individually for their attractiveness to different batches of larvae (Figure 10). These chemicals were indole, skatole, 2-propanol, ethanol, glycerol, methanol, butanol, p-cresol, (1S)-(-)-beta-pinene, and citral. The controls for the assay were as follows: an empty filter paper disc as a negative control and a filter paper disc with ground up rabbit food (normal larval diet) as a positive control. Early 3rd instar larvae were starved for 3 hours before being placed in the middle section on the starting “X” position. The larvae were able to roam freely about the tray for 10 minutes before their position in one of the three sections was documented.

2.8 Bacterial dsRNA Expression

RNAi induced knockdown in mosquito larvae was achieved using a bacterial dsRNA expression system. To knock down OR34, PCR primers targeting a 260 base pair region of the coding sequence spanning were ordered (IDT; Table 1). To knock down OR7, GeneBlocks (dsDNA gene fragments) targeting a 280 base pair region of the coding sequence were ordered (IDT; Table 1). DsRNA target sequences are listed in Table 2. Agarose gel electrophoresis was

performed on the PCR products and the annealed DNA oligonucleotides and bands of the correct anticipated size were excised from the gel. Gel extraction and purification was performed using the E.Z.N.A.® Gel Extraction Kit (www.omega.com). Gel purified constructs were blunt end ligated into the pJet 1.2 vector using T4 DNA ligase (www.thermofisher.com). The recombinant plasmids were used to transform competent *E. coli* DH5a cells, which were then plated on LB + ampicillin (Amp) agar plates. Individual colonies were selected and verified as having the desired gene fragment insert using a PCR-based colony screen using gene specific primers (Table 1) and agarose gel electrophoresis. After confirmation that the selected colonies contained the correct insert, they were grown overnight at 37ºC in a LB broth culture supplemented with ampicillin [100 μg/mL]. Plasmids were purified using the E.Z.N.A.® Plasmid DNA Mini Kit (www.omega.com).

DsRNA-target templates were excised out of pJet 1.2 using XbaI and XhoI restriction digests and the fragments were then ligated into the similarly digested pL4440 dsRNA expression vector using the T4 DNA ligase. The pL4440 plasmids containing the dsRNA-expression constructs were then transformed into a nuclease deficient and tetracycline resistant competent strain of *E. coli* cells, HT115. Cells were plated on LB + Amp + tetracycline (Tet) plates. Individual colonies were selected, and presence of the insert was verified as described above. Production of dsRNA in the bacterial cells was achieved by first growing up cultures overnight at 37ºC in a LB broth culture supplemented with amp [100 μg/mL] and tet [12.5 μg/mL]. Next, overnight cultures were added to 108 mL of LB with 108 μl of Amp and 108 μl of Tet in baffled flasks. Cultures were grown at 37ºC for 3-4 hours until reaching an optical density of 0.6. IPTG was then added to a concentration of 0.6 mM to induce production of the dsRNA. Cells grew for an additional 4 hours before being centrifuged at 3,750xg for 5 minutes and collected for bacterial food pellets

for knockdown assays. Negative controls consisted of pellets containing only yeast and agar (i.e. no bacteria) and a control with a randomly scrambled DNA sequence inserted into the plasmid.

Pelleted cells were resuspended in 3mL of Brewer's yeast (100mg/mL) and 12 mL of 1% warm agar. The mixture was placed in an 80ºC water bath for 10 minutes to heat-kill the bacteria. The solution was mixed and vortexed thoroughly before being poured into 5mL syringes with their tops cut off; the syringes holding the bacterial agar mixtures were allowed to cool and solidify. Syringes were covered and stored at 4ºC until needed.

2.9 Feeding Mosquito Larvae Bacteria that Express dsRNA

Through RNAi, transcript knockdown of OR34 and OR7 was performed using the bacterial-feeding assay. *Aedes aegypti* larvae were hatched and counted into groups of 10 before being placed in deep petri dishes along with roughly 200 mL of deionized water and two 0.5 mL dsRNA bacterial agar discs, cut from the syringes, as their sole source of nutrition. Pellets were replaced every 2 days or sooner if they were consumed quickly. The assay was performed with three biological replicates for each treatment at 22 ºC (room temperature). Apart from the insects used for bioassays, other batches of three groups of 10 larvae were allowed to grow until they reached the late 2nd instar stage, then allowed to feed on the bacterial-agar discs for just 2 days before they were collected, and their RNA was extracted (as described above) to assess the extent of knockdown using qRT-PCR. Whereas the larvae for the bioassays are hatched and fed on the bacterial-agar discs for 5 days before reaching the 3rd instar stage and then were subjected to the chemotaxis assay.

Table 2. **DsRNA target sequences**

Gene Name	**Accession**	**Sequence 5'-3'**
Aedes aegypti OR34	NM_001358623.1	TTTCAGTAGCCGGTCAGCAGTTGGATTTGCTCA GCTGTAAATTTGCCAGCCTGCCGGTTCCTGGAA CGGGAACCACTATTGAAAAAGACTATTATAAAA GGACGTTTGTGGAGCAGATTCAACATTTTGCACG GGAGATCGAGAGAACAGTTTCGTGGTGTGTTTTTG CACAGATATGTGCCAGCGGTATAACCATTTGCGCC ATAGTATTCCGTTTGTCGGCTATCAGTATTATCGAT CATCTCGGCACCTCAA
Aedes aegypti OR7	NM_001358471.1	AAATCTAGAACATTCTTCGGCGATAGCGTCAAAAA TGTATTCGACAAAGAGACTAATGAAACGTATACGG TGGAAATTCCCCGATTGCCCATCAAGGCTTGGTACC CGTGGGATGCAATGAGCGGAGTGCCGTACTTTTTCT CCTTCATCTACCAGGCTTATTTCCTGCTGTTTTCGAT GTGCCAGGCCAACCTCGCCGATGTGATGTTCTGCTC CTGGCTGCTCTTCACTTGCGAACAGCTGCAGCATTT GAAGGGTATAATGCGCCCCCTCGAGAAA

3 RESULTS

3.1 OR34 as a Target Gene

qRT-PCR analyses were used to examine transcript abundance across mosquito developmental stages. *Aedes aegypti* OR34 was found to be expressed across all mosquito life stages but was predominantly expressed in 1st instar larvae, with 125-fold greater transcript abundance in 1st instars relative to 2nd instars and 725-fold greater transcript abundance in 1st instars relative to adults (Figure 1). Tissue dissections performed on *Aedes aegypti* larvae revealed that OR34 is expressed in all larval tissues. It shows the highest level of transcript accumulation in the head but there was no statistical difference in its distribution in the rest of the body (Figure 2). This is consistent with the distribution of most of the olfactory tissues in adult mosquitoes, where the majority of the odorant receptor expression is in the head.

Aedes aegypti OR34 is a GPCR-like protein with multiple transmembrane domains. It is made up of 11 alpha-helical domains (Figure 3). A phylogenetic analysis was used to examine the predicted evolutionary relationships of OR34 proteins in insects (Figure 4). Using MEGAX, a maximum likelihood phylogenetic tree was constructed with 100 bootstrap replicates. A BLAST search identified 47 putative homologues of the *Aedes aegypti* OR34 protein in other insects, all of which were included in the tree. Notably, the OR34 of *Aedes aegypti* clusters more closely with that of the two *Culex* species than with *Aedes albopictus*, while OR34s in the more distantly related mosquito genus, *Anopheles*, clustered in four different clades, suggesting that the OR34 protein has undergone multiple divergences over the course of mosquito evolution.

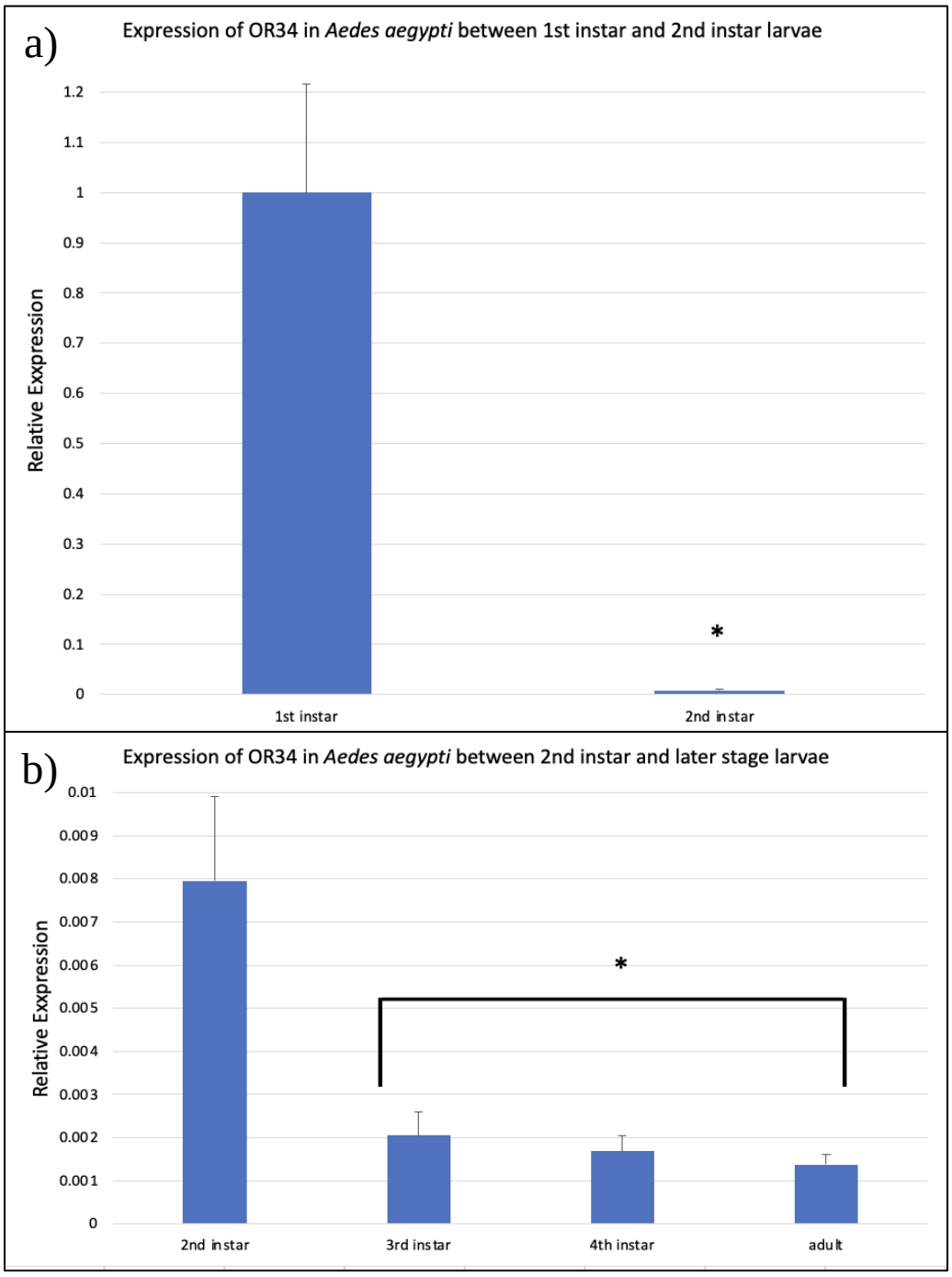

Figure 1. Relative Expression of OR34 in *Aedes aegypti* across developmental stages. Relative expression between 1st and 2nd instar larvae is shown in (a). As there is such a large difference in relative expression, for better visualization, panel b shows 2nd instar larvae through adult stages relative to the 1st instars. cDNA was obtained from three pools of 10 individuals. QRT-PCR was performed using primers from Table 1 and using *Ribosomal protein subunit 7 (s7)* as the reference gene as described in materials and methods. Statistical significance between treatments was determined using one-way ANOVA. * denotes significant differences in the comparison groups ($P<0.05$).

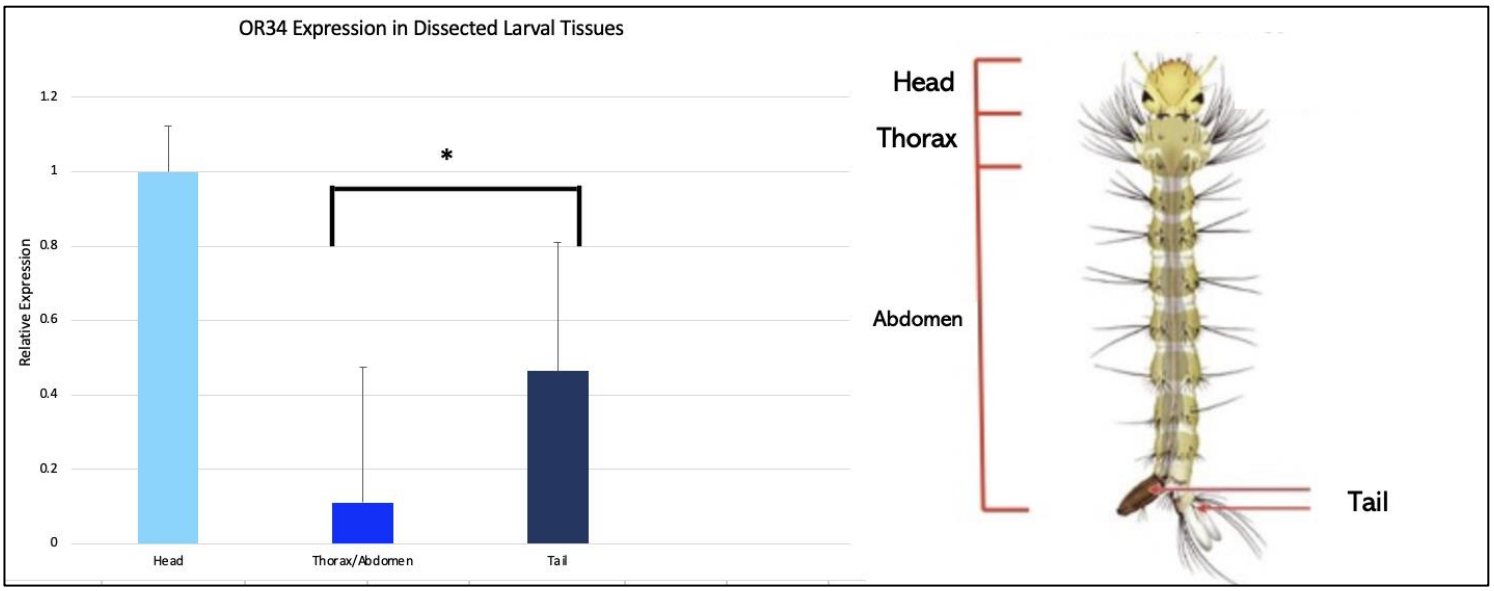

Figure 2. Relative Expression of OR34 in *Aedes aegypti* in dissected larvae. cDNA was obtained from three pools of 10 individual heads, thorax and abdomens, and tails. QRT-PCR was performed using primers from Table 1 and using *Ribosomal protein subunit 7 (s7)* as the reference gene as described in materials and methods. Statistical significance between treatments was determined using one-way ANOVA ($p<0.05$).

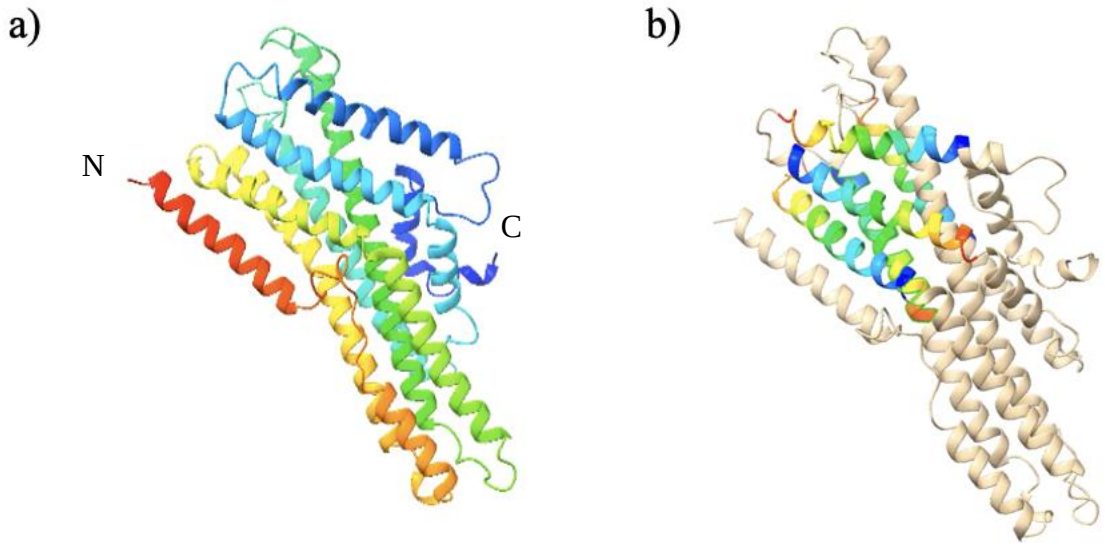

Figure 3. Predicted 3D structure of *Aedes aegypti* OR34 (a), with transmembrane domains highlighted in colour (b). Structures were predicted using Alphafold and drawn using ChimeraX.

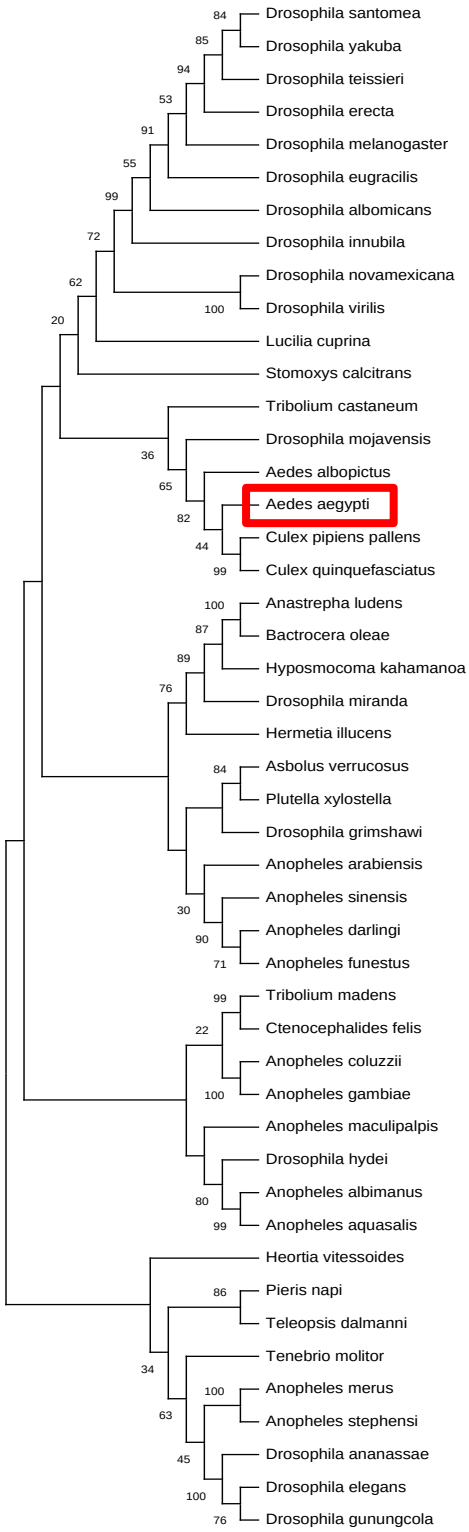

Figure 4. Phylogenetic tree of OR34 orthologs based on amino acid sequences. Maximum likelihood tree constructed using MEGA. *Aedes aegypti* is highlighted. Bootstrap support values above 20 displayed next to nodes.

3.2 Comparison of OR34 between *Aedes aegypti* and *Aedes albopictus*

As *Aedes aegypti* and *Aedes albopictus* are closely related species, we would expect homologs to have largely similar sequences and similar structures, but with some features unique to each species. An examination of the predicted structure of their proteins can potentially aid in identifying what ligands bind to various proteins, and comparisons of any changes in protein structures in two related species may help explain species-specific differences in their functions. In particular, differences in the extracellular domains between species can explain differences in their ability to bind a ligand (Supplemental Table 2). The *Aedes aegypti* and *Aedes albopictus* OR34 amino acid sequences are 83% identical (Figure 5) and their predicted structures, including the overall fold of the proteins, are also similar (Figure 6).

OR34 Amino Acid Alignment

```
Aae     MGEFDDFIPSQRVAFWIWKILGIWATDDESPFYRAYRRIYHFFFTGIYLFSMFTSSFFTE   60
Aal     MGQFDEFIPSQRAAFWIWKIYGIWATEDESPFYRTYRRVYHFLFTGVYLFSMFISSFFTE   60
        **:**:******.******* *****:*******:***:***:***:****** ******

Aae     NSEELWVEILFILPTEIAMLTKTIITVYKFETIHRLLQTTISKEFQPTCPKHGKEYDRFF   120
Aal     NSEELWGEILFILPTELAMLTKNVITVNKFETIYALHRTSISKEFQPTCPKHGKEYDLYF   120
        ****** *********:*****.:*** *****: * :*:****************** :*

Aae     DRFSKVMLMYYFCSVCAAWTHLGFLFDDRLKLPFFNWFFWVPLDRDHLNNYYILFAYQMI   180
Aal     DRFSKLMLMYFFVSICAATSHFGFLFDDRLKLPFFNWFFWVPLDRDHLNNYYLLFAYQMI   180
        *****:****:* *:*** :*:*********************************:*******

Aae     GMMGHCSLNVSGDMNIAYLLSVAGQQLDLLSCKFASLPVPGTGTTIEKDYYKRTFVEQIQ   240
Aal     GMMGHCCLNVSGDMNIAYLLSIAGQQLDLLSCKFACLPVPRAGTNAEKDHYRRKFVAQIQ   240
        ******.***************:**************.**** :**. ***:*:*.** ***

Aae     H------FAREIERTVSWCVFAQICASGITICAIVFRLSAISIIDHLGTSIPMFFYMVSM   294
Aal     QYNRIYEFARDIEKAVSWGVFAQICASGITICAIVFRLSSISIIDHLGTSIPMFFYMVSM   300
        :      ***:**::*** ********************:**********************

Aae     LTQIFLPCYFGNDVTLKSQKLTNALYTSKWYRLAMSDRKDLKMMTLRTSESIRLKAGGFF   354
Aal     LTQIFMPCYFGNDVTLKSQTLTNALYTSEWYQLAMDDRKDLKMMMLRTGESIRLKAGGFF   360
        *****:*************.********:**:***.******** ***.***********

Aae     NFNLEAFTSTLNTAYSVYAVLNSKNNK-   381
Aal     NFNLDAFTSTLNTAYSVYAVLNSQEKNK   388
        ****:********************::::
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Figure 5. Amino acid alignments of OR34 in *Aedes aegypti* and *Aedes albopictus.* Amino acid sequences obtained through NCBI were aligned using Clustal Omega (83% identity).

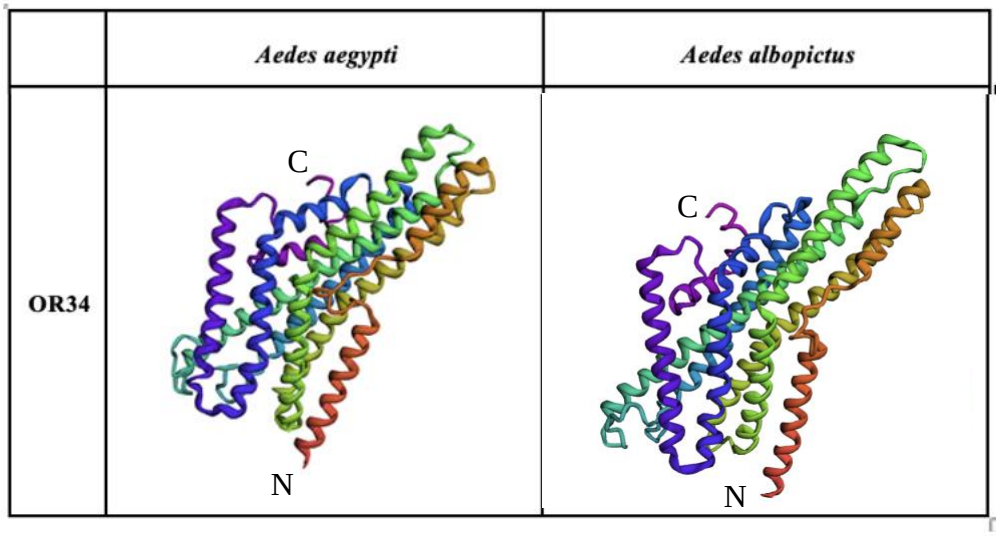


Figure 6. Comparison of the 3D predicted structures of OR34 in *Aedes aegypti* and *Aedes albopictus*. Structures were made through Robetta by uploading amino acid sequences from NCBI of the respective proteins.

3.3 Establishing Chemotaxis Assay Conditions

Prior to the chemotaxis assay, the chemicals to be challenged were prepared as described in the Materials and Methods. Chemicals belonging to the same chemical groups were mixed in equal volumes. Groups to be tested consisted of the indoles, ketones, carboxylic acids (COOH), alcohols and aldehydes 1 (Alc/Ald 1), alcohols and aldehydes 2 (Alc/Ald 2), and terpenes (Table 3).

To assess the sensitivity of the larvae to the chemicals, a dose response chemotaxis assay was performed (as described below). Concentrations of 0.1 mM, 0.01 mM, 0.001 mM, and 0.0001 mM were tested. For each of the pooled chemicals tested, 0.1 mM was the lowest concentration where the larvae could distinguish between the chemical and the control (Figure 8). This is consistent with the working concentration from a similar study (Huff and Pitts 2019) and is the concentration that was used for all further chemotaxis assays.

Table 3. Chemical group compositions for chemotactic assay. Chemicals were diluted to a concentration of 0.1 mM. Equal volumes of each individual chemical were then mixed with members belonging to the same chemical groups.

Indoles	Ketones	COOH	Alc/Ald 1	Alc/Ald 2	Terpenes
Skatole	(+/-)-camphor	Acetic acid	2-propanol	Anisole	(1S)-(-)-beta-pinene
Indole	6-methyl-5-hepten-2-one	Glycine	Ethanol	Cyclohexanol	Citral
	Acetone	Heptanoic acid	Glycerol	Hexanal	
	Coumarin	Hexanoic acid	Methanol		
	Ethyl acetate	Octanoic acid	Butanol		

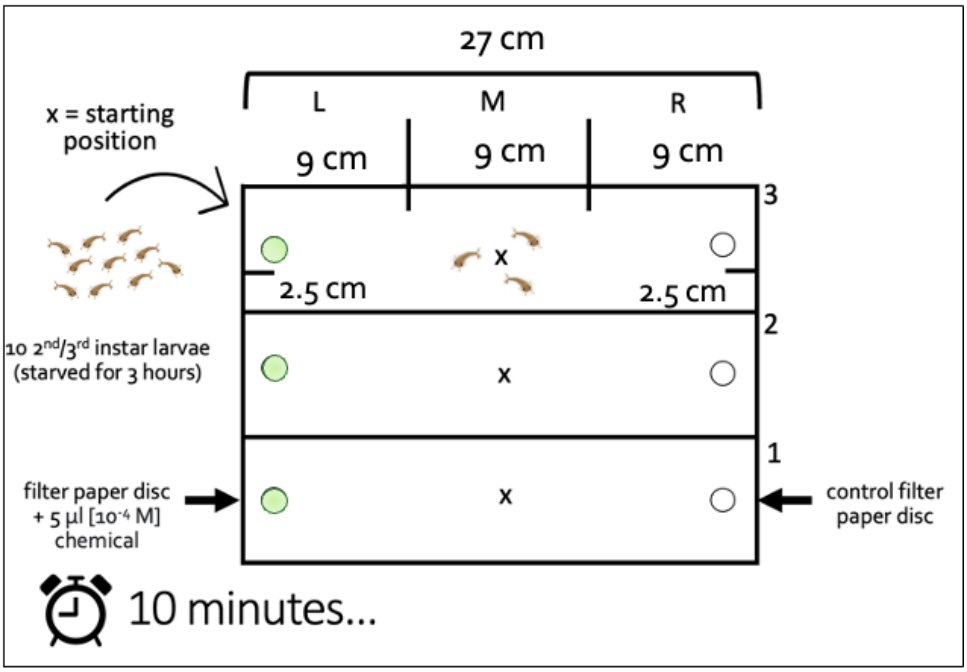


Figure 7. Chemotaxis Assay Set-Up. The assay was set up with 3 equal lanes 27 cm in length, divided into 3 equal sections of 9 cm. The sections were labelled "L", "M", and "R" for left, middle, and right, respectively. On both the left- and right-hand sides of the tray, a 1 cm diameter filter paper disc was placed 2.5 cm from the edge of the tray. On the left-hand side, the filter paper disc was saturated with 5 µL of a chemical with a concentration of 0.1mM. On the right-hand side, the filter paper disc was the control. Ten 3rd instar larvae were starved for 3 hours before being placed on the middle "X". The larvae roamed about the tray for ten minutes before their position in one of the three sections was documented.

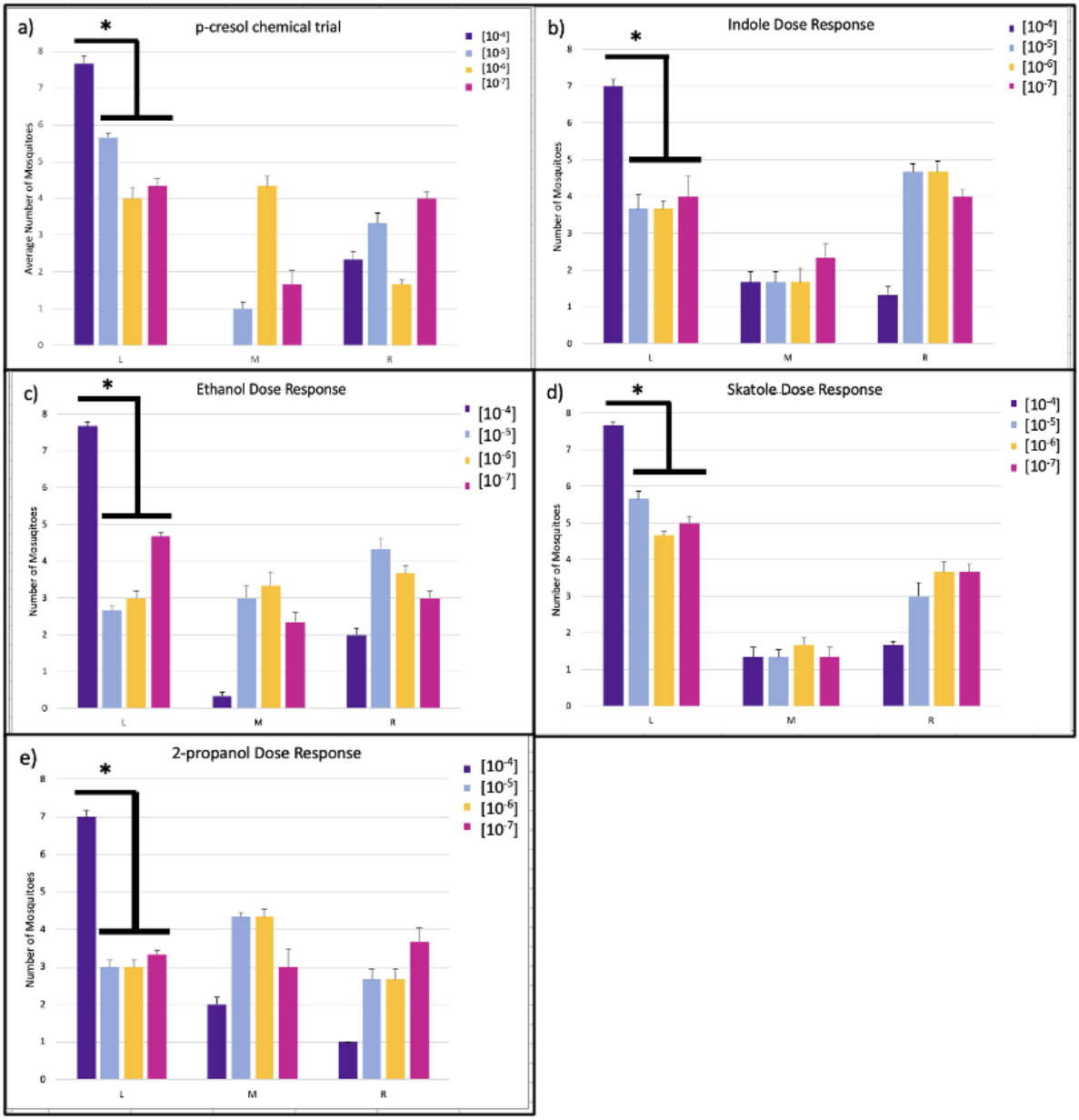


Figure 8. Dose response assay to test the sensitivity of *Aedes aegypti* larvae to attractive chemicals. 5 µL of chemicals (p-cresol (a), indole (b), ethanol (c), skatole (d), and 2-propanol (e)) ranging in concentrations from 0.0001 mM to 0.1 mM were used to challenge mosquito larvae as described in the chemotaxis assay. Statistical significance between treatments was determined using one-way ANOVA ($p<0.05$).

Initial assays focused on screening groups of chemicals, prepared according to Table 3. The groups tested were the indoles, ketones, carboxylic acids (COOH), alcohols and aldehydes 1 (Alc/Ald 1), alcohols and aldehydes 2 (Alc/Ald 2), and terpenes. The groups of chemicals that

attracted the highest proportion of larvae in these initial screens were the indoles, alcohols and aldehydes 1, and terpenes (Figure 9). These groups of chemicals were then broken down into their individual components and were tested in the chemotaxis assay individually in the same way (Figure 10).

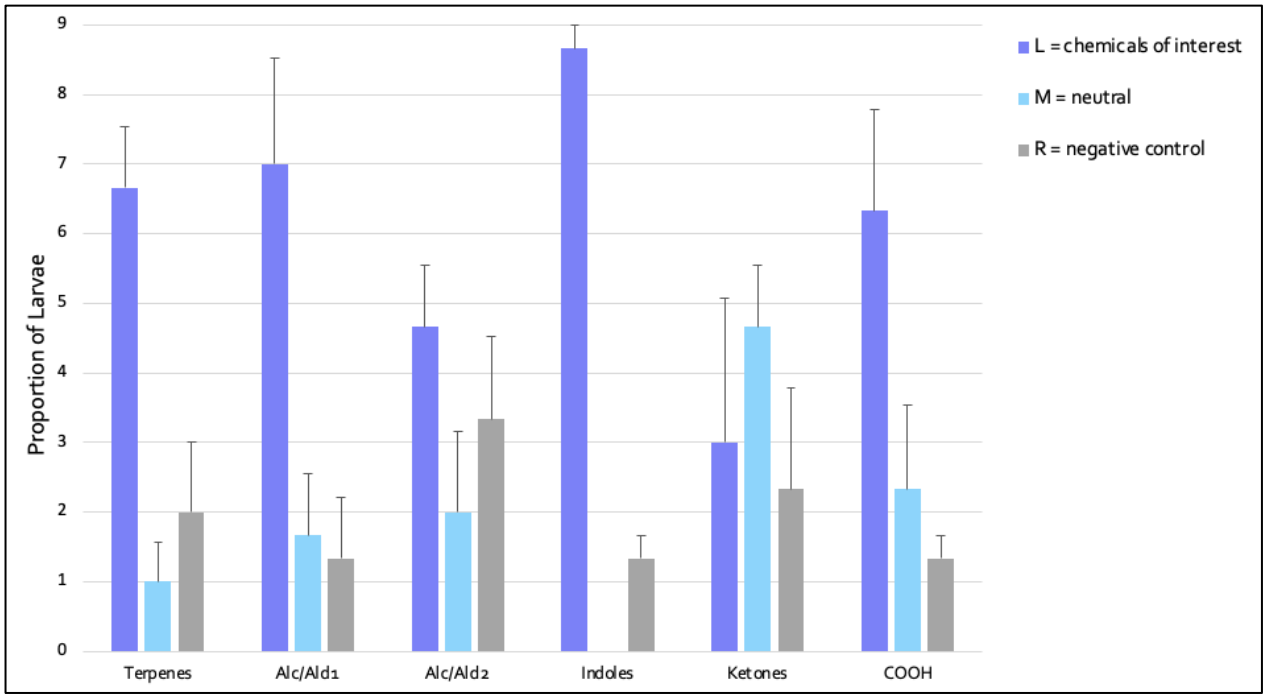


Figure 9. Chemotaxis Results for Initial Chemical Screens of Groups. Assay was done according to Figure 7. Chemical groups were prepared according to Table 3.

The individual chemicals tested in the chemotaxis assay after the initial chemical screens were indole, skatole, 2-propanol, ethanol, glycerol, methanol, butanol, citral, beta-pinene, and p-cresol (Figure 10). The chemicals showing the most attraction compared to the controls (were indole, skatole, 2-propanol, ethanol, and p-cresol. Therefore, these chemicals were those that were tested in the knockdown assays (Figure 11, Figure 17, Figure 19). These 5 chemicals were also used for in silico docking analyses and all 5 ligands were predicted to bind and have favourable interactions with the protein (Supplemental Figure 1).

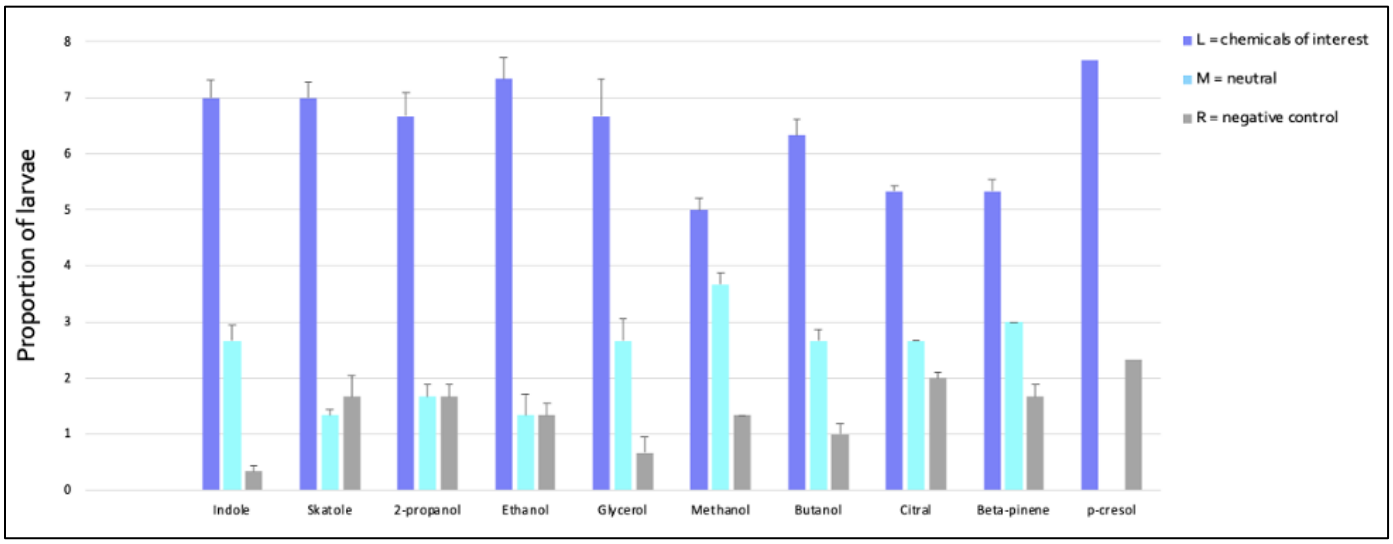


Figure 10. Chemotaxis Results for Individual Chemicals. Assay was done according to Figure 7. Chemical groups were prepared according to Table 3.

3.4 Impacts on larval response to attractive chemicals following consumption of bacterial dsRNA targeting OR34

In total, the responses to indole, 2-propanol, and ethanol were measured following 5 days of consumption of bacteria expressing OR34 dsRNA in comparison to their response to the same chemicals following consumption of the negative control bacteria, under the same conditions. These three chemicals were chosen out of the five as they have the most variation between themselves in where they would likely be found in the environment. Indole is commonly found in feces (Candeliere *et. al.* 2022), 2-propanol is a metabolic byproduct of many microorganisms (Vernocchi *et. al.* 2016) and ethanol is found in overripe fruit (Dudley 2004)

For the three chemicals tested, a reduction in attraction to the odorants compared to the control treatments was observed (Figure 11). qRT-PCR confirmed that the OR34 transcript levels were knocked down 93% after 2 days of feeding on the dsRNA-expressing bacteria, relative to the negative controls (Figure 12).

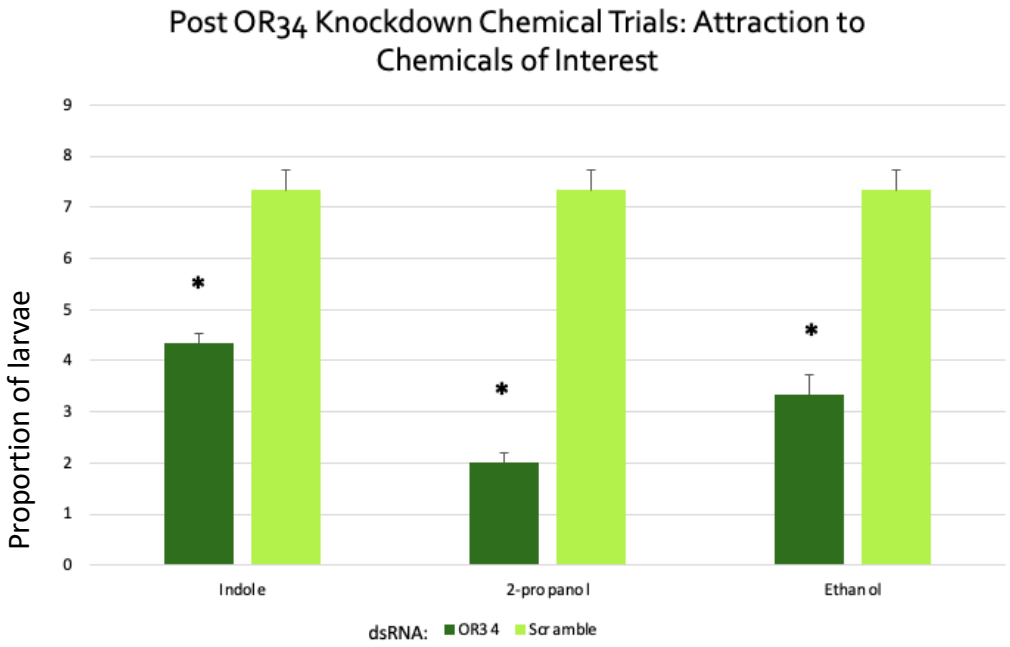


Figure 11. Post OR34 Knockdown Response to Attractive Chemicals. Response of larvae to attractive chemicals following feeding of bacterial pellets (OR34 knockdown or Scramble control) for 5 days to *Aedes aegypti* larvae from hatched to 3rd instar (according to Figure 7). The values represent the means and standard errors of the three replicates of 10 pooled larvae. Statistical significance was determined through ANOVA ($p<0.05$).

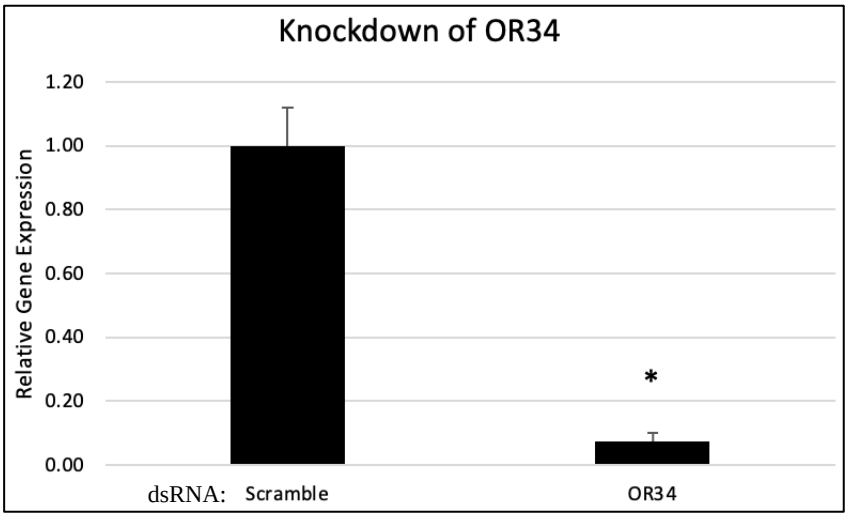


Figure 12. OR34 Knockdown. Through qRT-PCR using *Aedes aegypti* 40s ribosomal protein (s7) as a reference gene, 93% knockdown of *Aedes aegypti* OR34 was observed. Larvae were grown up until the 3rd instar stage before being fed on bacterial food pellets containing the OR34 dsRNA for 2 days. The values represent the means and standard errors of the three replicates of 10 pooled larvae. The asterisk indicates a statistically significant difference in the transcript abundances (ANOVA, $P<0.05$).

3.5 The role of ORCO (OR7)

To further validate this bioassay, we examined the effect of knocking down expression of ORco. Since ORco pairs with every other odorant receptor to detect odorants, it plays a critical role in mosquitoes' olfactory systems. In mosquitoes, and other insect species, ORco is highly conserved (Figure 16). The *Aedes aegypti* and *Aedes albopictus* OR7 amino acid sequences are 98% identical (Figure 13). Their predicted structures are very similar with the proteins shoqing the same overall fold (Figure 14). ORco was found to be expressed across all developmental stages, confirmed via qRT-PCR (Figure 15).

The chemotaxis assay and the OR7 dsRNA bacterial pellets were set-up and prepared in the same way as the OR34 assay and pellets (Figure 7). Since there was a limited amount of OR7 bacterial pellets, the chemicals tested for this validation assay were narrowed down from the initial 5 to just 2. Indole and p-cresol were chosen as these would be highly likely to be found in the larva's natural environment. Indole is widespread in the environment but is typically found near plants or bacteria (Ferrer *et. al.* 2022; Ma *et. al.* 2018) while p-cresol is a metabolic intermediate produced by bacteria in the gut by humans and animals. Both p-cresol and indole are products of bacterial metabolism from amino acid fermentation and are excreted in feces (Candeliere *et. al.* 2022). For the chemicals tested we observed a reduction in attraction to the odorants compared to the control treatments (Figure 17). Using qRT-PCR, OR7 transcripts were found to be knocked down 86.5%, relative to the negative controls, following 2 days of consumption of the bacteria expressing OR7 dsRNA (Figure 18).

OR7 Amino Acid Alignment

```
Aae       MNVQPTKYHGLVLDLMPNIRLMQGFGHFLFRYVNGPVLIRKLYSWWNLIMILLQYFAIMG   60
Aal       MNVQPTKYHGLVLDLMPNIRLMQGFGHFLFRYVSGPVLIRKLYSWWNLIMILLQYFAIMG   60
          ********************************.**************************

Aae       NLVMNTGDVNELTANTITTLFFTHSVTKFIYVAVNSEHFYRTLGIWNQPNSHSLFAESDA   120
Aal       NLVMNTGDVNELTANTITTLFFTHSVTKFIYVAVNSEHFYRTLGIWNQPNSHSLFAESDA   120
          ************************************************************

Aae       RYHSIALAKMRKLLVMVMVTTVLSVVAWITITFFGDSVKNVFDKETNETYTVEIPRLPIK   180
Aal       RYHSIALAKMRKLLVMVMVTTVLSVVAWITITFFGDSVKNVFDKETNETYTVEIPRLPIK   180
          ************************************************************

Aae       AWYPWDAMSGVPYFFSFIYQAYFLLFSMCQANLADVMFCSWLLFTCEQLQHLKGIMRPLM   240
Aal       ALYPWDAMSGVPYFFSFVYQAYFLLFSMCQANLADVMFCSWLLFTCEQLQHLKGIMRPLM   240
          * ***************:********************************************

Aae       ELSATLDTYRPNSAALFRVASAGSKSELILNEEKDPDTKDFDLNGIYNSKADWGAQFRAP   300
Aal       ELSASLDTYRPNSAALFRAASAGSKAELILNEEKDPDTKDFDLNGIYNSKADWGAQFRAP   300
          ****:**************.******:**********************************

Aae       STLQTFGD-NGINGNPNGLTKKQELMVRSAIKYWVERHKHVVRLVSAIGETYGAALLLHM   359
Aal       STLQTFNDNNGMNGNPNGLTKKQELMVRSAIKYWVERHKHVVRLVSAIGETYGAALLLHM   360
          ******.* **:************************************************

Aae       LTSTIKLTLLAYQATKIDALNVYGLTVIGYLVYALAQVFLFCIFGNRLIEESSSVMEAAY   419
Aal       LTSTIKLTLLAYQATKIDALNVYGLTVIGYLVYALAQVFLFCIFGNRLIEESSSVMEAAY   420
          ************************************************************

Aae       SCHWYDGSEEAKTFVQIVCQQCQKAMTISGAKFFTVSLDLFASVLGAVVTYFMVLVQLK    478
Aal       SCHWYDGSEEAKTFVQIVCQQCQKAMTISGAKFFTVSLDLFASVLGAVVTYFMVLVQLK    479
          ************************************************************
```

Figure 13. Amino acid alignments of OR7 in *Aedes aegypti* and *Aedes albopictus.* Amino acid sequences obtained through NCBI were aligned using Clustal Omega (98% identity).

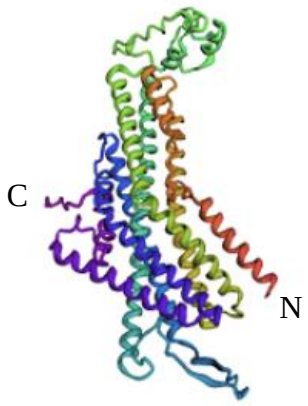
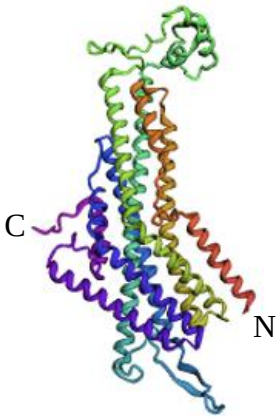
	Aedes aegypti	*Aedes albopictus*
OR7		

Figure 14. Comparison of the 3D predicted structures of OR7 in *Aedes aegypti* and *Aedes albopictus.* Structures were made using Robetta by uploading amino acid sequences from NCBI of the respective proteins.

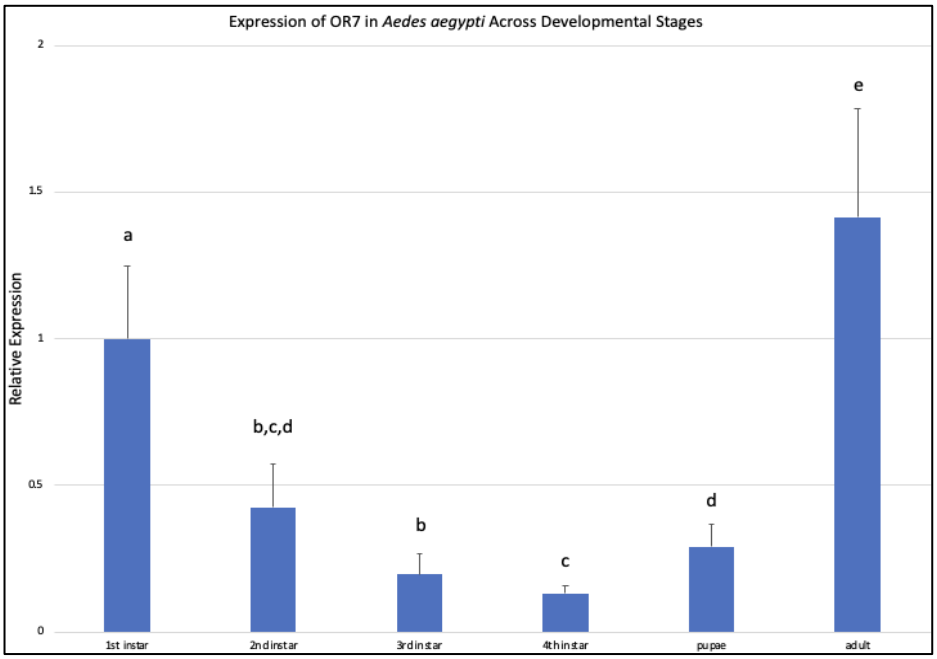


Figure 15. Relative Expression of OR7 in *Aedes aegypti* across developmental stages. cDNA was obtained from pools of 10 individuals. QRT-PCR was performed using primers from Table 1 and using with *Ribosomal protein subunit 7 (s7)* as the reference gene as described in materials and methods. Statistical significance between treatments was determined using one-way ANOVA ($p<0.05$).

A maximum likelihood phylogenetic tree was also constructed for OR7 using MEGAX with 100 bootstrap replicates (Figure 16). A BLAST search was performed on the *Aedes aegypti* OR7 proteins. For this analysis, only select species of interest with a high percentage of sequence similarity were included in the tree. For OR7, *Aedes aegypti* clusters closest with *Anopheles gambiae* rather than with its sibling species, *Aedes albopictus*, and its other more closely related mosquito, *Culex quinquefasciatus*.

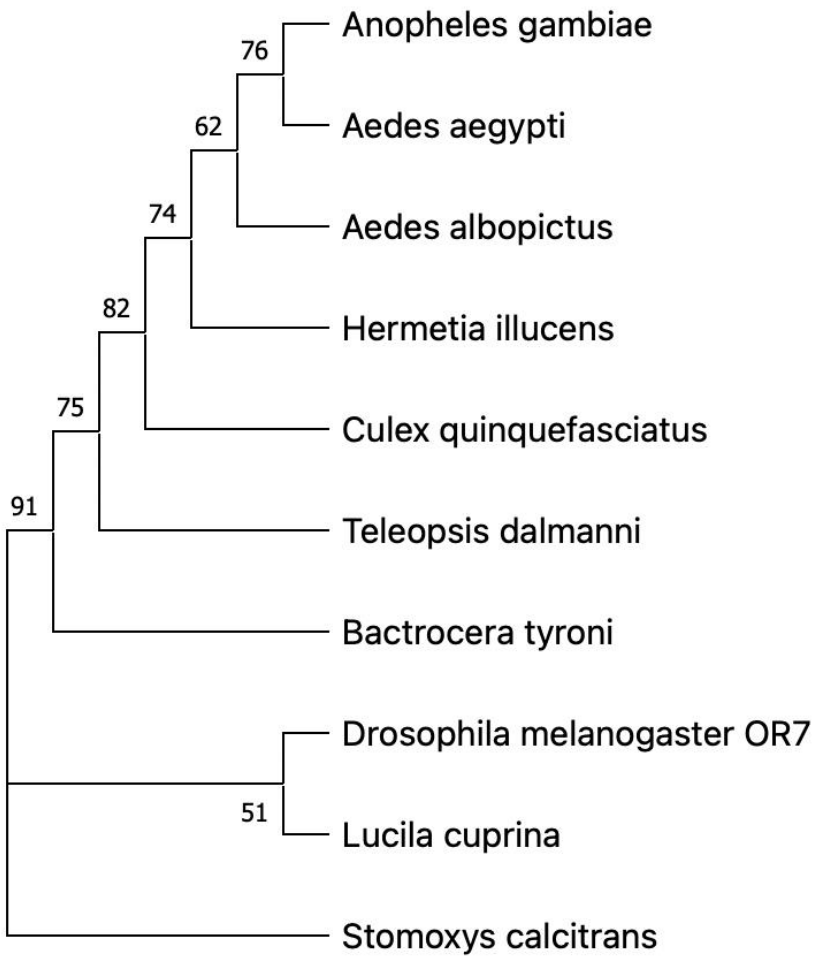


Figure 16. Phylogenetic tree of OR7 orthologs based on amino acid sequences. Tree constructed using MEGA. Bootstrap support values above 20 displayed next to nodes.

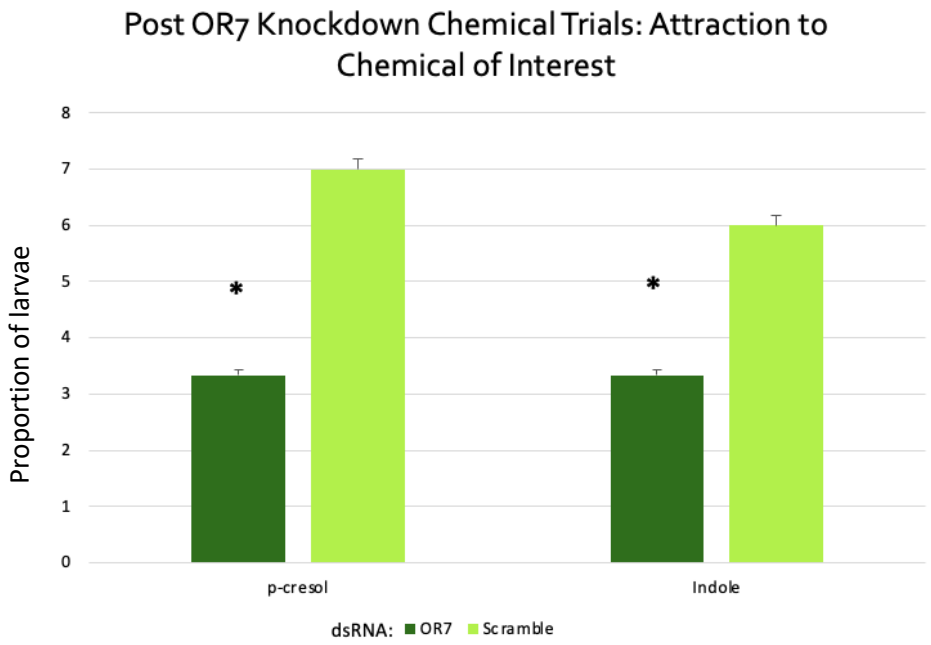


Figure 17. Post OR7 Knockdown Response to Attractive Chemicals. Response of larvae to attractive chemicals following feeding of bacterial pellets (OR7 knockdown or Scramble control) for 5 days to *Aedes aegypti* larvae from hatched to 3rd instar (according to Figure 7). The values represent the means and standard errors of the three replicates of 10 pooled larvae. Statistical significance was determined through ANOVA ($p<0.02$).

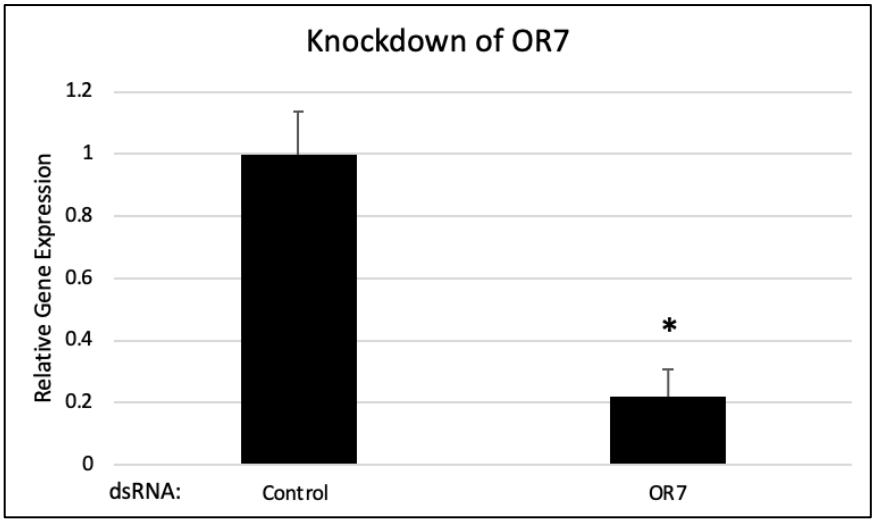


Figure 18. OR7 Knockdown. Through qRT-PCR using *Aedes aegypti* 40s ribosomal protein (s7) as a reference gene, 86.5% knockdown of *Aedes aegypti* OR7 was observed. Larvae were grown until the 3rd instar stage before being fed on bacterial food pellets containing the OR7 dsRNA for 2 days. The values represent the means and standard errors of the three replicates of 10 pooled larvae. The asterisk indicates a statistically significant difference in the transcript abundances (ANOVA, $P<0.05$).

3.6 Impact of a Double Knockdown Assay

To assess whether a double-knockdown assay of both odorant receptors would have a larger impact than the individual OR knockdown on the ability of the larvae to detect various odorants, bacterial pellets containing both OR34 dsRNA and OR7 dsRNA (double-knockdown pellets) were prepared as described in the materials and methods. After consuming the double-knockdown pellets for 5 days, the larvae were subjected to the same chemotactic assay, with the same chemicals. For all the chemicals, we observed a reduction in the larvae's attraction compared to the control treatment (Figure 19). However, contrary to what was expected, we did not observe a larger reduction in attraction to the chemicals after consuming the double OR knockdown pellets compared to the single OR knockdown pellets (ANOVA; $P<0.1$).

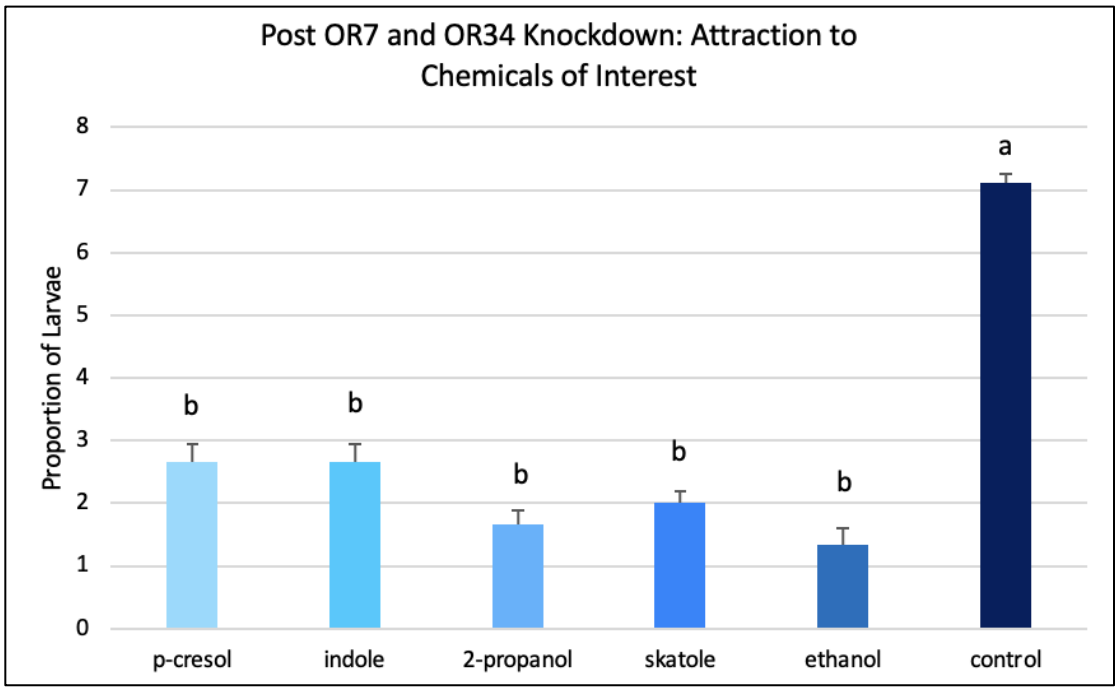


Figure 19. Post Double Knockdown (OR34 and OR7) Response to Attractive Chemicals. Response of larvae to attractive chemicals following feeding of bacterial pellets (OR34 + OR7 knockdown or Scramble control) for 5 days to *Aedes aegypti* larvae from hatched to 3rd instar (according to Figure 7). Chemicals along the X axis refer to the larvae's response to the respective chemical after consuming OR34 + OR7 knockdown bacterial food pellets. Statistical significance between treatments was determined using one-way ANOVA ($p<0.05$).

4 DISCUSSION

With olfaction playing such a critical role in mosquitoes' abilities to carry out many of their fundamental survival and reproductive processes, identifying ways to disrupt this complex sensory system is key for developing novel control strategies. Through the use of bioinformatics and a chemotaxis response assay, chemicals attractive to *Aedes aegypti* larvae were identified. One odorant receptor, OR34, was identified as the focus of this study as it is predominantly larvally expressed and has an unknown ligand. Through dsRNA-mediated knockdown of OR34, three putative ligands of the receptor were identified. To further validate the assay, ORco was knocked down and a similar reduction in attraction to the odorants was observed. To assess whether simultaneously knocking down more than one OR has a greater effect than knocking down one OR individually, a double knockdown assay was done. Dual knockdown of OR34 and ORco together resulted in a reduction in chemoattraction similar to the individual knockdown of either OR34 or ORco alone. Based on these preliminary findings, it is not yet possible to conclude whether an additive or synergistic effect can be achieved by knocking down these two receptors together. However, by refining the assay further, using different doses of dsRNA and/or odorants, it may be possible to identify which ORs are critical for food seeking behaviours in these mosquito larvae.

4.1 Identification of Potential Ligands for *Aedes aegypti* OR34

In *Aedes aegypti*, 131 odorant receptors have been identified, but for many of these odorant receptors, their functions and what ligands they bind remain unclear. Identifying which odorants bind to which OR could provide critical information to develop effective control methods. The dsRNA feeding experiments provide evidence that the chemicals tested could be

potential ligands of OR34. However, this study focused on testing only a limited number of compounds. Chemicals were initially screened as groups of similar chemicals before being separated and tested individually. In this manner, the list of potential ligands could be quickly narrowed down. For future studies, it would be worthwhile to explore a wider range of chemicals in the RNAi-mediated knockdown experiments to conclusively identify the receptor's ligand(s). Rather than just randomly screening hundreds of potential ligands, it would be more efficient to first use bioinformatics programs to investigate which ligands are most likely to bind to the protein before doing any bioassays. In this study, the SwissDock program was used to explore whether a small subset of chemicals that were attractive to the larvae could bind to a protein similar to OR34 protein. Because *Aedes aegpyti* OR34 has not had its structures experimentally confirmed, the *Machilis hrabei* OR5 was used as the closest approximation. Using this proxy molecule, *in silico* modeling suggested that this protein would bind all the odorants that were confirmed to be attractive to the mosquito larvae (Supplemental Figure 1). The fact that these *in silico* analyses confirmed the RNAi knockdown results suggests that the use of the *M. hrabei* OR5 could serve as a suitable test protein for more in silico analyses of OR34. However, since the docking showed similar results for all the ligands tested, the results from this analysis were not conclusive.

Overall, this study's results were unable to discriminate the most favoured odorant ligand, as they all showed potential to bind to the protein. Instead of conducting any further chemotaxis experiments, it would be more informative to take an approach like that of Caballero-Vidal *et. al.* in 2020, where they used machine learning to virtually screen 3 million odorants for their potential to bind SlitOR25, an odorant receptor in *Spodoptera littoralis*, a crop pest insect species. Through their initial screens, 32 molecules were identified as potential matches, which

were later tested on the SlitOR25. Of these 32 odorants, 11 were revealed to be novel agonists of the receptor, using single sensillum electrophysiological tests. Caballero-Vidal *et. al.* emphasize the importance of their results in optimizing the discovery of novel agonists, as well as comparing the chemical structures of these agonists to analyze the structure-function relationship between the receptor and its ligands. Similarly, Boyle *et. al.* in 2013, Yuan *et. al.* in 2019, and Alfonso-Prieto and Capelli in 2023, all explored using in silico bioinformatic approaches to screen large numbers of candidate ligands to predict potential receptor-ligand interactions before narrowing down the list of odorants to be tested in functional assays. For this research, it would have been a useful strategy to perform the docking analyses as well as other computational predictive analyses before beginning any bioassays. This would have allowed many more odorants to be initially screened. Overall, this would have been a quick and efficient method leading to more conclusive evidence when functionally validating odorants.

The chemicals that were tested share some common features with one another. For example, three of the chemicals: indole, skatole, and p-cresol, share cyclic structures (PubChem n.d.). The other two chemicals: ethanol and 2-propanol are alcohols and so they contain an "-OH group", also shared by p-cresol as it is a phenol derivative (PubChem n.d.). All the compounds are relatively small molecules. Aside from sharing some structural similarities, the compounds also share similarities in where they are typically found in the environment. P-cresol is a microbial metabolite that is found in mammalian feces, urine, and sweat (Candeliere *et. al.* 2022; MarkerDB n.d.). In humans, it is produced through bacterial fermentation of protein (Hamer *et. al.* 2012). P-cresol is also a major component of pig, elephant, and horse volatile odors (Putmai *et. al.* 2023; Wheeler *et. al.* 1982; Būda *et. al.* 2013). Next, indole is a widely distributed chemical in the environment. It is a metabolite produced by many types of bacteria when

degrading tryptophan (MarkerDB n.d). Like p-cresol, indole is commonly found in feces (Candeliere *et. al.* 2022). It is also found in plants such as orange blossoms, jasmine, and fermented Bermuda grass infusions (MarkerDB n.d;Dekel *et. al.* 2022). Like indole, skatole is found in the feces of animals and is produced by the same plants (Zgarbova and Vrzal 2023). Additionally, it is also produced as a metabolite during tryptophan metabolism in the digestive tract through bacterial activity. Next, 2-propanol is a metabolic byproduct of numerous microorganisms (Vernocchi *et. al.* 2016) and small amounts can be made in a human's body (MarkerDB n.d.). This compound is highly volatile meaning that it could be a component of human sweat. It has also been detected in certain fresh produce such as papaya, apples, onions, and cherries (MarkerDB n.d.). Finally, ethanol can also be found in rotting or overripe fruit as well as in certain plants (Dudley 2004). It is a byproduct of yeast metabolism and can be found in human sweat (MarkerDB n.d; Maicas 2020). Therefore, as we would expect, the odorants that are attractive to mosquitoes all share the characteristic of involving being near food sources (bacteria for mosquito larvae) or animal sources (for mosquito adults). Most of them are found in some type of bodily secretion of either humans or other animals. Exploring where compounds are commonly found and their relevance to the organism being studied at their specific life stage is another screening method that can be used in hand with the information from bioinformatics analysis to provide important context and help with making an informed selection of trial chemicals before performing the bioassay.

Finally, this research focused on identifying putative ligands of *Aedes aegypti* OR34 because the *Aedes albopictus* colony died before any functional assays could be performed. However, a bioassay comparing the two species' responses to the same ligands would be valuable information to have. Since their sequences and their predicted structures were

compared, exploring whether this has an impact on their responses to the same chemicals would provide insight into how small changes in sequence or structure can change the functionality of the receptor. For example, Jiang *et. al.* in 2015 and Trimmer *et. al.* in 2018 explored how small changes in sequences of ORs in humans resulted in differences in how they perceived particular odorants, as well as modified the receptor's sensitivity and specificity to compounds. In insects, the evolution of ORs is not totally clear. However, we do know that there was rapid diversification of ORs across species, indicating that perhaps few changes in sequence were necessary for the emergence of unique ORs, attuned to their own unique odorants (Tian *et. al.* 2022). It would also be interesting to perform the chemotaxis assay with the same chemicals in some of the other closely related mosquito species such as *Culex quinquefasciatus* and *Culex pipiens pallens*, as these two species clustered more closely together to *Aedes aegypti* than *Aedes albopictus* in phylogenetic analyses of the OR34 protein. Overall, this research provided some insight into putative ligands of *Aedes aegypti* OR34, but it is possible that its most biologically relevant ligand has not yet been identified and more detailed analyses are still needed.

4.2 Chemotaxis Assay and Other Olfactory Driven Mosquito Control Methods

In this study a chemotaxis assay similar to that of Huff and Pitts in 2019 was used to challenge *Aedes aegypti* larvae with an array of potentially attractive chemicals. This relatively easy assay can be used both for fundamental research and could lead to some interesting mosquito control applications. In this study, this assay was used in basic research to explore the response of the larvae to the odorants after knocking down a specific OR to attempt to identify its ligand. However, the assay can also provide useful information to develop larval traps for use in mosquito control methods. Larval traps work by luring the larvae into the trap with attractive

odorants (Jahir *et. al.* 2022). Once inside the trap, the larvae are unable to escape and will eventually die. There are also similar traps designed to target egg-laying adult mosquitoes. These devices work by attracting gravid mosquitoes to a trap where they will lay their eggs. The mosquito eggs will hatch as they normally would, but the mosquitoes will be unable to get food or escape the trap and will also die. These traps are simple and require only water, a small mesh net, and an alluring compound, often using sugar-fermenting yeast as a CO_2 source (Jahir *et. al.* 2022). Finally, larvicides are another larval control method that function similarly by using attractive chemicals to lure the larvae to a certain area. But instead of simply trapping the larvae and waiting until they die, they intentionally kill them through the use of bacterial or chemical larvicides (Zhou *et. al.* 2020). Overall, this chemotaxis assay provides valuable information that can be used in many different control applications which can easily be applied to control the larvae of other pest insect species.

This research did not examine olfaction in adult mosquitoes but many of the concepts explored could be similarly applied in adult insects. Instead of the chemotaxis assay being performed in an aquatic environment, a Y-shaped olfactometer could be used. Y-shaped olfactometers are instruments that are used in bioassays where a tunnel branches into 2 distinct channels and the mosquito must choose which path to take, similar to how the chemotaxis assay used in this study permitted the larvae to move to the left or right side of the tray (Yu *et. al.* 2015). And in the same way that attractive chemicals can be used to bait larvae into traps, attractive chemicals can also be used to lure and trap adult mosquitoes. Overall, researching and understanding an insect's olfactory system at all its developmental stages can be a very useful tool in bettering or developing control methods.

4.3 Identifying a Suitable Odorant Receptor to Target for Larval Control Applications

Different levels of expression across developmental stages in the two ORs tested in this chemotaxis system were observed. *Aedes aegypti* OR34 is expressed primarily in 1st instar larvae. In contrast, *Aedes aegypti* OR7 is expressed across all life stages. Despite their differences in expression levels, individually knocking down either OR34 or OR7 both resulted in a reduction of the larvae's attraction to previously attractive chemicals. This could indicate that knocking down more than one odorant receptor at a time is not necessary in order to achieve the desired effect and so the OR targeted for knockdown assays should be carefully selected. Since OR7 is the co-receptor that functions along with every other OR, it seems like the most obvious choice to target for mosquito control applications. This has shown to be somewhat successful in adult *Anopheles gambiae and Aedes aegypti* mosquitoes, where interfering with ORco resulted in an extreme reduction in their attraction to certain compounds (DeGennaro *et. al.* 2013). Both species show a large preference to feeding on human hosts. However, silencing ORco by RNAi is not absolute, and in the presence of CO_2, the mosquitoes retained their ability to detect hosts. Interestingly, they did however lose their ability to discriminate between host species, meaning that they no longer preferred humans to other hosts. Other studies have demonstrated that knocking down ORco also impacts mosquito's abilities to locate food, mates, and oviposition sites (Sun *et. al.* 2020; Liu *et. al.* 2016). However, in mosquito larvae, as they are aquatic, the presence or absence of CO_2 is not likely the primary attractant for food-seeking larvae.

This research demonstrated that knocking down ORco in mosquito larvae can be an effective method at reducing their ability to detect attractive odorants. Few studies have examined the effect of knocking down ORco in larvae but Sun *et. al.* in 2020 used CRISPR to create an ORco knockout mutant in *Anopheles coluzzii* larvae. They observed that ORco mutants were unable to detect common odorant, including indole and skatole, which were found to be attractive to *Aedes aegypti* larvae in my study. Not surprisingly, the suite of odorants detected by larvae and adult mosquitoes differed, but nevertheless, the loss of ORco resulted in substantial disruptions to the different-staged mosquitoes' abilities to find food.

It is also important to consider that RNAi typically does not result in total loss of function of the targeted gene. It only reduces the transcript's abundance, producing a knockdown effect and not a total knockout. The relative degree to which a gene can be knocked down varies depending on the species and the gene. It is also not totally clear how long the effects of RNAi last in mosquitoes. In some insect species, the gene silencing effect is temporary while in others the effect persists and can even affect the second generation (Joga *et. al.* 2016; Huvenne and Smagghe 2009; Baum *et. al.* 2007). These are important factors to evaluate when choosing a target gene and choosing what life stage to induce knockdown. This, along with the many redundancies already built into the mosquito's olfactory system indicates that it may be necessary to target more than one type of receptor at the same time to achieve the desired phenotype.

In this research we looked at the effect of knocking down OR34 and ORco simultaneously. Although we did not observe any significant differences between knocking down both ORs and knocking down one of the individual ORs, this does not indicate that targeting more than one olfactory receptor together is not worthwhile. Although there have not

been many studies exploring knocking down an OR and an IR, an OR and a GR, and an IR and a GR, Sparks *et. al.* in 2018 note that this would be an interesting avenue to explore.

5 CONCLUSIONS

This research explored the impact that disrupting a component of a mosquito's olfactory system can have on their ability to properly perform one of their critical functions – detection of potential food sources. In this study, a predominantly larvally expressed odorant receptor, OR34, was targeted and putative ligands of this receptor were identified. To further validate the assay, the impact of ORco on mosquito olfaction was also tested. Knockdown of OR34 and OR7 together produced similar results to individually knocking down either OR, suggesting that more research is needed to understand the impacts of knocking down more than one OR at a time has on the mosquito behaviour and survival. Research into control methods targeting the insect at the larval stage is a growing area of interest and interfering with their olfactory systems could complement existing methods of mosquito control based on chemical pesticides.

This project describes a chemotaxis response assay that is an easy and effective way to screen for attractants and could be applied in control methods for many other pest aquatic species. By identifying chemical attractants, larval traps can be employed by luring larvae with an attractive compound, so they are unable to escape. Another option for larval control is supplying and feeding the larvae with food containing dsRNA targeting the knockdown of specific ORs, similar to the knockdown assay done with bacterial food pellets. Interfering with the mosquitoes' olfactory systems at the larval stage could slow their development sufficiently to prevent the mosquitoes from developing into blood-feeding adults.

Future studies should aim to explore whether knocking down OR34 and ORco in *Aedes albopictus* has the same reduction in attraction to the same chemicals as observed in *Aedes*

aegypti. While this research identified a subset of attractive chemicals for mosquito larvae, there are numerous other chemicals that remain to be explored. Future research should also explore the impact of knocking down a number of ORs at the same time to assess whether this leads to a stronger reduction in the mosquito's ability to detect odorants. However, since there are likely many ORs involved in food-seeking behaviours, it may be more useful to knock down one of the olfactory system's co-receptors instead such as ORco or one of the three IR co-receptors: IR8a, IR25a, and IR76b for greater impact. Employing the chemotaxis and knockdown assays with other pest insect species with aquatic stages such as black flies, horse flies, and deer flies, would be an interesting next step. Finally, designing a similar chemotaxis assay that could accurately identify attractive compounds for adult insects would be a useful step in developing new control methods for adult insect populations.

Together, this research sheds some light on an efficient and effective way of identifying putative ligands for a specific OR. Further developing this assay and applying it in different ways to different species will enhance our understanding of insect olfaction as well as aid in the development of insect control methods at various stages of development.

6 REFERENCES

Ababasi, R.., Heschuk, D., Kim, B. Whyard, S. A novel paperclip double-stranded RNA. structure demonstrates clathrin-independent uptake in the mosquito *Aedes aegypti. Insect. Biochem. Mol. Bio.* **127,** 103492 (2020). https://doi.org/10.1016/j.ibmb.2020.103492

Afify, A., Betz, J.F., Riabinina, O., Lahondere, C., Potter, C.J. Commonly Used Insect Repellents Hide Human Odors from *Anopheles* Mosquitoes. *Curr. Biol.* **29**(21) 3668-3680 (2019). https://doi.org/10.1016/j.cub.2019.09.007

Aktar, M.W., Sengupta, D., Chowdhury, A. Impact of pesticides use in agriculture: their benefits and hazards. *Interdisc Toxicol.* **2**(1) 1-12 (2009). doi: 10.2478/v10102-009-0001-7

Alberts, B., Johnson, A. D., Lewis, J., Morgan, D., Raff, M. C., Roberts, K., & Walter, P. (2014) *Molecular Biology of the Cell*. W. W. Norton.

Alfonso-Prieto, M., Capelli, R. Machine Learning-Based Modeling of Olfactory Receptors in Their Inactive State: Human OR51E2 as a Case Study. *J. Chem. Inf. Model.* **63,** 2911-2917 (2023). https://doi.org/10.1021/acs.jcim.3c00380

Alonso San Alberto, D., Rusch, C., Zhan, Y. *et al.* The olfactory gating of visual preferences to human skin and visible spectra in mosquitoes. *Nat Commun* **13,** 555 (2022). https://doi.org/10.1038/s41467-022-28195-x

Alphey, L., Wise de Valdez MR, Nimmo D, Betz J, Gong HF, James AA, Black WC 4th. Genetic elimination of dengue vector mosquitoes. Proc Natl Acad Sci U S A. 2011 Mar 22;108(12):4772-5. doi: 10.1073/pnas.1019295108. (2011)

American Mosquito Control Association. (2020). Control.

https://www.mosquito.org/page/control

Aryan, A., Anderson, M.A.E., Biedler, J.K., Qi, Y., Overcash, J.M., Naumenko, A.N., Sharakhova, M. V., Mao, C., Adelman, Z.N., Tu, Z., Nix alone is sufficient to convert female Aedes aegypti into fertile males and myo-sex is needed for male flight. *Proc. Natl. Acad.*. **117**, 17702–17709. (2020). https://doi.org/10.1073/pnas.2001132117

Athrey, G., Popkin-Hall, Z., Cosme, L.V. *et al.* Species and sex-specific chemosensory gene expression in *Anopheles coluzzii* and *An. quadriannulatus* antennae. *Parasites Vectors* **13,** 212 (2020). https://doi.org/10.1186/s13071-020-04085-3

Batra, S., Corcoran, J., Zhang, D., Pal,P., K.P, U., Kulkarni, R., Lofstedt, C., Sowdhamini, R., Olsson, S. A Functional Agonist of Insect Olfactory Receptors: Behaviour, Physiology and Structure. *Front. Cell. Neurosci.*13:134 (2019). doi: 10.3389/fncel.2019.00134

Baum, J., Bogaert, T., Clinton, W. *et al.* Control of coleopteran insect pests through RNA interference. *Nat Biotechnol* **25,** 1322–1326 (2007). https://doi.org/10.1038/nbt1359

Belluco S, Bertola M, Montarsi F, Di Martino G, Granato A, Stella R, Martinello M, Bordin F, Mutinelli F. Insects and Public Health: An Overview. Insects. 2023 Feb 27;14(3):240. doi: 10.3390/insects14030240. PMID: 36975925; PMCID: PMC10059202.

Bohbot, J.D., Dickens, J.C. (2009) Characterization of an Enantioselective Odorant Receptor in the Yellow Fever Mosquito *Aedes aegypti*. PLOS ONE **4**(9) (2009). https://doi.org/10.1371/journal.pone.0007032

Bohbot, J., Pitts, R.J., Kwon, H-W., Rützler, M., Robertson, H.M., Zwiebel, L.J. Molecular characterization of the *Aedes aegypti* odorant receptor gene family. *Insect Molecular Biology* **16**(5), 525–537 (2007). doi: 10.1111/j.1365-2583.2007.00748.x

Boyle SM, McInally S, Ray A. Expanding the olfactory code by in silico decoding of odor-

receptor chemical space. Elife. 2013 Oct 1;2:e01120. doi: 10.7554/eLife.01120.

Būda, V., Mozūraitis, R., Kutra, J. *et al. p*-Cresol: A Sex Pheromone Component Identified from the Estrous Urine of Mares. *J Chem Ecol* **38**, 811–813 (2012). https://doi.org/10.1007/s10886-012-0138-2

Caballero-Vidal G, Bouysset C, Grunig H, Fiorucci S, Montagné N, Golebiowski J, Jacquin-Joly E. Machine learning decodes chemical features to identify novel agonists of a moth odorant receptor. Sci Rep. 2020 Feb 3;10(1):1655. doi: 10.1038/s41598-020-58564-9.

Candeliere F, Simone M, Leonardi A, Rossi M, Amaretti A and Raimondi S (2022), Indole and p- cresol in feces of healthy subjects: Concentration, kinetics, and correlation with microbiome.Front. Mol. Med. 2:959189. doi: 10.3389/fmmed.2022.959189

Cappelle, K., de Oliveira, C. F., Van Eynde, B., Christiaens, O., and Smagghe, G. The involvement of clathrin-mediated endocytosis and two Sid-1-like transmembrane proteins in double-stranded RNA uptake in the Colorado potato beetle midgut. *Insect Mol. Biol.* **25**, 315–323. (2016). doi: 10.1111/imb.12222

Centers for Disease Control and Prevention. (2020). *Ae. aegypti* and *Ae. albopictus* mosquitoes. https://www.cdc.gov/mosquitoes/about/life-cycles/aedes.html

Centers for Disease Control and Prevention. (2022). Genetically Modified Mosquitoes. https://www.cdc.gov/mosquitoes/mosquito-control/community/emerging-methods/genetically-modified-mosquitoes.html

Centers for Disease Control and Prevention. (2018). Yellow Fever. https://www.cdc.gov/globalhealth/newsroom/topics/yellowfever/index.html

Christophers, S.R., 1960. Aëdes Aegypti (L.), the Yellow Fever Mosquito: Its Life History, Bionomics, and Structure. Cambridge University Press, Cambridge.

Das De, T., Thomas, T., Verma, S., Singla, D., Chauhan, C., Srivastava, V., Sharma, P., Kumari, S., Tevatiya, S., Rani, J., Hasija, Y., Pandey, KC., Dixit, R. A Synergistic Transcriptional Regulation of Olfactory Genes Drives Blood-Feeding Associated Complex Behavioral Responses in the Mosquito *Anopheles culicifacies. Front. Physiol.* **9**;577 (2018). doi: 10.3389/fphys.2018.00577

DeGennaro M, McBride CS, Seeholzer L, Nakagawa T, Dennis EJ, Goldman C, Jasinskiene N, James AA, Vosshall LB. orco mutant mosquitoes lose strong preference for humans and are not repelled by volatile DEET. Nature. 2013 Jun 27;498(7455):487-91. doi: 10.1038/nature12206.

DeGennaro M. The mysterious multi-modal repellency of DEET. Fly (Austin). 2015;9(1):45-51. doi: 10.1080/19336934.2015.1079360. PMID: 26252744; PMCID: PMC4594586.

Dekel A, Sar-Shalom E, Vainer Y, Yakir E, Bohbot JD. The ovipositor cue indole inhibits animal host attraction in Aedes aegypti (Diptera: Culicidae) mosquitoes. Parasit Vectors. 2022 Nov 12;15(1):422. doi: 10.1186/s13071-022-05545-8.

Dudley R. Ethanol, fruit ripening, and the historical origins of human alcoholism in primate frugivory. Integr Comp Biol. 2004 Aug;44(4):315-23. doi: 10.1093/icb/44.4.315.

Dusfour I, Chaney SC. Mosquito control: Success, failure, and expectations in the context of arbovirus expansion and emergence. Mosquitopia: The Place of Pests in a Healthy World [Internet].; 2022. 14. https://www.ncbi.nlm.nih.gov/books/NBK585173/doi: 10.4324/9781003056034-19

Erdelyan, C.N., Mahood, T.H., Bader, T.S., Whyard, S. Functional validation of the carbon dioxide receptor genes in Aedes aegypti mosquitoes using RNA interference. *Insect Mol Biol*. **21**(1), 119-127. (2011). doi: 10.1111/j.1365-2583.2011.01120.x.

European Centre for Disease Prevention and Control. (2022). Mosquito Factsheets. https://www.ecdc.europa.eu/en/disease-vectors/facts/mosquito-factsheets

Famakinde DO. Public health concerns over gene-drive mosquitoes: will future use of gene-drive snails for schistosomiasis control gain increased level of community acceptance? Pathog Glob Health. 2020 Mar;114(2):55-63. doi: 10.1080/20477724.2020.1731667. Epub 2020 Feb 26. PMID: 32100643; PMCID: PMC7170313.

Ferrer L, Mindt M, Suarez-Diez M, Jilg T, Zagorščak M, Lee JH, Gruden K, Wendisch VF, Cankar K. Fermentative Indole Production via Bacterial Tryptophan Synthase Alpha Subunit and Plant Indole-3-Glycerol Phosphate Lyase Enzymes. J Agric Food Chem. 2022 May 11;70(18):5634-5645. doi: 10.1021/acs.jafc.2c01042.

Giordano, B.V., Gasparotto, A., Liang, P., Nelder, M.P., Russell, C., Hunter, F.F. Discovery of an Aedes (Stegomyia) albopictus population and first records of Aedes (Stegomyia) aegypti in Canada. *Med. Vet. Entomol*. **34**(1):10-16. (2020). doi: 10.1111/mve.12408.

Hall, R.D., Gerhardt, R.R. Flies (Diptera). *Medical and Veterinary Entomology.* (2002). https://doi.org/10.1016/B978-0-12-510451-7.X5000-2

Hammond, A., Galizi, R., Kyrou, K. *et al.* A CRISPR-Cas9 gene drive system targeting female reproduction in the malaria mosquito vector *Anopheles gambiae*. *Nat Biotechnol* **34,** 78–83 (2016). https://doi.org/10.1038/nbt.3439

Hamer, H., De Preter, V., Windey, K., Verbeke, K. Functional analysis of colonic bacterial metabolism: relevant to health? Amer. *J. Phys. Gastro. Liver. Phys.* **302**, (2012). https://doi.org/10.1152/ajpgi.00048.2011

Harris, A., Nimmo, D., McKemey, A. *et al.* Field performance of engineered male mosquitoes.*Nat Biotechnol* **29**, 1034–1037 (2011). https://doi.org/10.1038/nbt.2019

Harris, A., McKemey, A., Nimmo, D. *et al.* Successful suppression of a field mosquito population by sustained release of engineered male mosquitoes. *Nat Biotechnol* **30**, 828–830 (2012). https://doi-org.uml.idm.oclc.org/10.1038/nbt.2350

Henle, A. (2019, Jan. 24). Ted-Ed. *How CRISPR lets you edit DNA.* [Video]. YouTube. https://www.youtube.com/watch?v=6tw_JVz_IEc

Huff RM, Pitts RJ (2019) An odorant receptor from Anopheles gambiae that demonstrates enantioselectivity to the plant volatile, linalool. PLoS ONE 14(11): e0225637. https://doi.org/10.1371/journal.pone.0225637

Huvenne H, Smagghe G. Mechanisms of dsRNA uptake in insects and potential of RNAi for pest control: a review. J Insect Physiol. 2010 Mar;56(3):227-35. doi: 10.1016/j.jinsphys.2009.10.004.

Jahir A, Kahamba NF, Knols TO, Jackson G, Patty NFA, Shivdasani S, Okumu FO, Knols BGJ. Mass Trapping and Larval Source Management for Mosquito Elimination on Small Maldivian Islands. Insects. 2022 Sep 2;13(9):805. doi: 10.3390/insects13090805.

Jiang Y, Matsunami H. Mammalian odorant receptors: functional evolution and variation. Curr Opin Neurobiol. 2015 Oct. 34:54-60. doi: 10.1016/j.conb.2015.01.014.

Joga, M.R., Zotti, M.J., Smagghe, G., Christiaens, O. RNAi Efficiency, Systemic Properties, and Novel Delivery Methods for Pest Insect Control: What We Know So Far. *Front. Physiol*. (2016). https://doi.org/10.3389/fphys.2016.00553

Kent, L.B., Walden, .K.K., Robertson, H.M.,The Gr Family of Candidate Gustatory and Olfactory Receptors in the Yellow-Fever Mosquito *Aedes aegypti*. *Chemical Senses,* **33**(1) 79–93 (2007). https://doi.org/10.1093/chemse/bjm067

Konopka, J.K., Task, D., Aify, A., Raji, J., Deibel, K., Maguire, S., Lawrence, R., Potter, C.J.

Olfaction in *Anopheles* mosquitoes. *Chemical Senses* **46**, 1-24 (2021). DOI: 10.1093/chemse/bjab021

Kraemer, M., Sinka, M., Duda, K., Mylne, A., Shearer, F., & Barker, C. et al. (2015). The global distribution of the arbovirus vectors Aedes aegypti and Ae. albopictus. *Elife*, **4**. doi: 10.7554/elife.08347

Kythreoti G, Sdralia N, Tsitoura P, Papachristos DP, Michaelakis A, Karras V, Ruel DM, Yakir E, Bohbot JD, Schulz S, Iatrou K. Volatile allosteric antagonists of mosquito odorant receptors inhibit human-host attraction. *J Biol Chem*. (2021). doi: 10.1074/jbc.RA120.016557.

Leal, W. S., Choo, Y. M., Xu, P., da Silva, C. S., & Ueira-Vieira, C. Differential expression of olfactory genes in the southern house mosquito and insights into unique odorant receptor gene isoforms. *Proceedings of the National Academy of Sciences of the United States of America,* **110 (**46), 18704–18709. (2013). https://doi.org/10.1073/pnas.1316059110

Li, M., Yang, T., Bui, M. *et al.* Suppressing mosquito populations with precision guided sterile males. *Nat Commun* **12,** 5374 (2021). https://doi.org/10.1038/s41467-021-25421-w

Liu, H., Liu, T., Xie, L. *et al.* Functional analysis of Orco and odorant receptors in odor recognition in *Aedes albopictus* . *Parasites Vectors* **9**, 363 (2016). https://doi.org/10.1186/s13071-016-1644-9

Liu, N. Insecticide Resistance in Mossquitoes: Impact, Mechanisms, and Research Directions. *Annu. Rev. Entomol*. **60,** 537-559 (2015). https://doi.org/10.1146/annurev-ento-010814-020828

Liu, F., Wang, Q., Xu, P. *et al.* A dual-target molecular mechanism of pyrethrum repellency

against mosquitoes. *Nat Commun* **12,** 2553 (2021). https://doi.org/10.1038/s41467-021-22847-0

Lopez, S.B.G., Guimarães-Ribeiro, V., Rodriguez, J.V.G. *et al.* RNAi-based bioinsecticide for *Aedes* mosquito control. *Sci Rep* **9,** 4038 (2019). https://doi.org/10.1038/s41598-019-39666-5

Lutrat, C., Giesbrecht, D., Marois, E., Whyard, S., Baldet, T., Bouyer, J., Sex Sorting for Pest Control: It's Raining Men! *Trends Parasitol*. **35**, 649–662. (2019). https://doi.org/10.1016/j.pt.2019.06.001

Ma, Q., Zhang, X., Qu, Y. Biodegradation and Biotransformation of Indole: Advances and Perspectives. *Front. Microbiol.* **(9)**, (2018). https://doi.org/10.3389/fmicb.2018.02625

Maicas S. The Role of Yeasts in Fermentation Processes. Microorganisms. 2020 Jul 28;8(8):1142. doi: 10.3390/microorganisms8081142.

MarkerDB. (n.d.) https://markerdb.ca

Matthews, B.J. *Aedes aegypti*. *Trends Genetics.* **35**(6), 470-471. (2019). https://doi.org/10.1016/j.tig.2019.03.005

Mayo Clinic. (2018, July 24). *CRISPR Explained* [Video]. YouTube. https://www.youtube.com/watch?v=UKbrwPL3wXE

McBride, C., Baier, F., Omondi, A. *et al.* Evolution of mosquito preference for humans linked to an odorant receptor. *Nature* **515,** 222–227 (2014). https://doi-org.uml.idm.oclc.org/10.1038/nature13964

McMeniman CJ, Corfas RA, Matthews BJ, Ritchie SA, Vosshall LB. Multimodal integration of carbon dioxide and other sensory cues drives mosquito attraction to humans. Cell. (2014) 156(5):1060-71. doi: 10.1016/j.cell.2013.12.044

Meghani, Z. Regulation of genetically engineered (GE) mosquitoes as a public health tool: a public health ethics analysis. *Global Health* **18**, 21 (2022). https://doi.org/10.1186/s12992-021-00760-x

Mitra, S., Pinch, M., Kandel, Y., Li, Y., Rodriguez, S.D., Hansen, I.a. Olfaction-Related Gene Expression in the Antennae of Female Mosquitoes From Common Aedes aegypti Laboratory Strains. *Front. Physiol.* (2021). doi: 10.3389/fphys.2021.668236

Montell C. Gustatory receptors: not just for good taste. *Current Biology* **23**(20), 929–932 (2013). https://doi.org/10.1016/j.cub.2013.09.026

Munawar, K., Alahmed, A.M., Khalil, S.M. Delivery Methods for RNAi in Mosquito Larvae, *Journal of Insect Science*, **20**(4). (2020). https://doi.org/10.1093/jisesa/ieaa074

Muñoz-López, M., & García-Pérez, J. L. DNA transposons: nature and applications in genomics. *Current genomics*, **11**(2), 115–128 (2010). https://doi.org/10.2174/138920210790886871

National Environment Agency. (2020). Male Mosquitoes Do Not Bite or Transmit Disease. https://www.nea.gov.sg/corporate-functions/resources/research/wolbachia-aedes-mosquito-suppression-strategy/male-mosquitoes-do-not-bite

Nature Video. (2017, Oct. 31). *CRISPR: Gene editing and beyond* [Video]. Youtube. https://www.youtube.com/watch?v=4YKFw2KZA5o

Ni, M., Zhao, T., Lv, Hx. *et al.* Screening for odorant receptor genes expressed in *Aedes aegypti* involved in host-seeking, blood-feeding and oviposition behaviors. *Parasites Vectors* **15,** 71 (2022). https://doi.org/10.1186/s13071-022-05196-9

Pan American Health Organization. (2022). Annual Epidemiological Update for Dengue,

Chinkungunya and Zika in 2020. https://www3.paho.org/data/index.php/en/mnu-topics/indicadores-dengue-en/annual-arbovirus-bulletin-2020.html

Pelletier, J., Guidolin, A., Syed, Z. *et al.* Knockdown of a Mosquito Odorant-binding Protein Involved in the Sensitive Detection of Oviposition Attractants. *J Chem Ecol* **36,** 245–248 (2010). https://doi.org/10.1007/s10886-010-9762-x

PubChem. (n.d.) https://pubchem.ncbi.nlm.nih.gov

Purcell, A. Almeida, R. Insects as Vectors of Disease Agents. *Plant Crop Sci.* (2005). DOI: 10.1081/E-EPCS-120010496.

Putmai, N., Woraruthai, T., Tirapanampai, C., Wongnate, T., Flood, A. Biodegradation characteristics of mixed phenol and p-cresol contaminants from a swine farm using bacteria immobilized in calcium alginate beads. *Bioresour. Technol*. **23,** 101528. (2023). https://doi.org/10.1016/j.biteb.2023.101528

Ponlawat, A., Harrington, L.C., Blood Feeding Patterns of *Aedes aegypti* and *Aedes albopictus* in Thailand. *Journal of Medical Entomology*, **42**(5); 844–849. (2005). https://doi.org/10.1093/jmedent/42.5.844

Powell, J. Mosquito-Borne Human Viral Diseases: Why *Aedes aegypti*? *Am. J. Trop. Med. Hyg*. **98**(6), 1563-1565. (2018). https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6086192/

Pridgeon, J. W., Zhao, L., Becnel, J. J., Strickman, D. A., Clark, G. G., and Linthicum, K. J. Topically applied AaeIAP1 double-stranded RNA kills female adults of *Aedes aegypti*. *J. Med. Entomol.* **45**, 414–420. (2008). doi: 10.1603/0022-2585(2008)45[414:TAADRK]2.0.CO;2

Raji, J., Melo, N., Castillo, J., Gonzalez, S., Saldana, V., Stensmyr, M., & DeGennaro, M. Aedes

aegypti Mosquitoes Detect Acidic Volatiles Found in Human Odor Using the IR8a Pathway. *Current Biology*, **29(**8), 1253-1262.e7. (2019). doi: 10.1016/j.cub.2019.02.045

Resnik DB. Ethical issues in field trials of genetically modified disease-resistant mosquitoes. Dev World Bioeth. 2014 Apr;14(1):37-46. doi: 10.1111/dewb.12011. Epub 2012 Dec 21. PMID: 23279283; PMCID: PMC3620724.

Richards SL, Byrd BD, Reiskind MH, White AV. Assessing Insecticide Resistance in Adult Mosquitoes: Perspectives on Current Methods. Environ Health Insights. 2020 Sep 2;14:1178630220952790. doi: 10.1177/1178630220952790. PMID: 32952401; PMCID: PMC7477762.

Rinker, D.C., Pitts, J., Zhou, X., Suh, E., Rokas, A., Zwiebel, L.J. Blood meal-induced changes to antennal transcriptome profiles reveal shifts in odor sensitivities in *Anopheles gambiae*. PNAS **110**(20) 8260-2265. (2013). https://doi.org/10.1073/pnas.1302562110

Robbins, P.J., Cherniack, M.G., Review of the biodistribution and toxicity of the insect repellent *N*,*N*-diethyl-*m*-toluamide (DEET). *Toxic. Evir, Heal*. **18**(4) 503-525 (1986). https://doi.org/10.1080/15287398609530891

Rogers, K. Aedes. *Encyclopedia Britannica*. (2019). https://www.britannica.com/animal/Aedes

Rubin GM, Spradling AC. Genetic transformation of Drosophila with transposable element vectors. Science. 1982 Oct 22;218(4570):348-53. doi: 10.1126/science.6289436. PMID: 6289436.

San Alberto, D.A., Rusch, C., Zhan, Y., Straw, A.D., Montell, C., Riffell, J.A. The olfactory gating of visual preferences to human skin and visible spectra in mosquitoes. *Nature Communications* **13,** (555) (2022). https://doi.org/10.1038/s41467-022-28195-x

Schuh, T. (n.d.). American Museum of Natural History. *Which animal group makes up the largest percentage of the world's organisms?* https://www.amnh.org/explore/ology/earth/ask-a-scientist-about-our-environment/which-animal-group-has-the-most-organisms

Sparks JT, Botsko G, Swale DR, Boland LM, Patel SS, Dickens JC. Membrane Proteins Mediating Reception and Transduction in Chemosensory Neurons in Mosquitoes. Front Physiol. 2018 Sep 20;9:1309. doi: 10.3389/fphys.2018.01309.

Stanczyk, N., Brookfield, J., Ignell, R., Logan, J., Field, L. Behavioral insensitivity to DEET in *Aedes aegypti* is a genetically determined trait residing in changes in sensillum function. *PNAS* **107** (19), 8575-8580. (2010). https://doi.org/10.1073/pnas.1001313107

Strobl F, Anderl A, Stelzer EH. A universal vector concept for a direct genotyping of transgenic organisms and a systematic creation of homozygous lines. Elife. 2018 Mar 15;7:e31677. doi: 10.7554/eLife.31677.

Sun H, Liu F, Ye Z, Baker A, Zwiebel LJ. Mutagenesis of the orco odorant receptor co-receptor impairs olfactory function in the malaria vector Anopheles coluzzii. Insect Biochem Mol Biol. 2020 Dec;127:103497. doi: 10.1016/j.ibmb.2020.103497

Tayler, A., Heschuk, D., Giesbrecht, D., Park, J., & Whyard, S. Efficiency of RNA interference is improved by knockdown of dsRNA nucleases in tephritid fruit flies. *Open Biology*, **9**(12), 190198. (2019). doi: 10.1098/rsob.190198

Tian J, Dewer Y, Hu H, Li F, Yang S, Luo C. Diversity and Molecular Evolution of Odorant Receptor in Hemipteran Insects. Insects. 2022 Feb 21;13(2):214. doi: 10.3390/insects13020214.

Tomoyasu, Y., Miller, S. C., Tomita, S., Schoppmeier, M., Grossmann, D., and Bucher, G.

Exploring systemic RNA interference in insects: a genome-wide survey for RNAi genes in *Tribolium*. *Genome Biol.* **9**(1). (2008). doi: 10.1186/gb-2008-9-1-r10

Ulvila, J., Parikka, M., Kleino, A., Sormunen, R., Ezekowitz, R. A., Kocks, C., et al. Double-stranded RNA is internalized by scavenger receptor-mediated endocytosis in Drosophila S2 cells. *J. Biol. Chem.* 281, 14370–14375. (2006). doi: 10.1074/jbc.M513868200

United States Environmental Protection Agency. (n.d.). Success in Mosquito Control: An Integrated Approach. https://www.epa.gov/mosquitocontrol/success-mosquito-control-integrated-approach

Vernocchi, P., Chierico, F., Putignani, L. Gut microbiota profiling: metabolomics based approach to unravel compounds affecting human health. *Front. Microbiol*. **7**, (2016). https://doi.org/10.3389/fmicb.2016.01144

Vogel, E., Santos, D., Mingels, L. Verdonckt, T., Broeck, J. RNA Interference in Insects: Protecting Beneficials and Controlling Pests. *Front. Physiol.* **9,** 1912 (2019). https://doi.org/10.3389/fphys.2018.01912

Wheeler JW, Rasmussen LE, Ayorinde F, Buss IO, Smuts GL. Constituents of temporal gland secretion of the African elephant,Loxodonta africana. J Chem Ecol. 1982 May;8(5):821-35. doi: 10.1007/BF00994782.

Wheelwright M, Whittle CR, Riabinina O. Olfactory systems across mosquito species. Cell Tissue Res. 2021 Jan;383(1):75-90. doi: 10.1007/s00441-020-03407-2.

Whyard, S., Singh, A.D., Wong, S. Ingested double-stranded RNAs can act as species-specific insecticides. *Insect Biochem Mol Biol*. **39**(11) 824-32. (2009). doi: 10.1016/j.ibmb.2009.09.007.

Whyard, S., Erdelyan, C.N., Partridge, A.L. *et al.* Silencing the buzz: a new approach to

population suppression of mosquitoes by feeding larvae double-stranded RNAs. *Parasites Vectors* **8,** 96 (2015). https://doi.org/10.1186/s13071-015-0716-6

Whyard, S., Erdelyan, C.N., Partridge, A.L. *et al.* Silencing the buzz: a new approach to population suppression of mosquitoes by feeding larvae double-stranded RNAs. *Parasites Vectors* **8,** 96 (2015). https://doi.org/10.1186/s13071-015-0716-6

Wicher, D., Miazzi, F. Functional properties of insect olfactory receptors: ionotropic receptors and odorant receptors. *Cell Tissue Res* **383,** 7–19 (2021). https://doi.org/10.1007/s00441-020-03363-x

Wise de Valdez, M.R., Nimmo, D., Betz, J., Gong, Hong-Fei, Alphey, L., Black, W.C. Genetic elimination of dengue vector mosquitoes. *PNAS.* **108**(12) 4772-4775 (2011). https://doi.org/10.1073/pnas.1019295108

World Health Organization. (2022). Dengue and severe dengue. https://www.who.int/news-room/fact-sheets/detail/dengue-and-severe-dengue

World Health Organization. (2022). Malaria. https://www.who.int/news-room/fact-sheets/detail/malaria

Wunderlich S, Gatto KA. Consumer perception of genetically modified organisms and sources of information. Adv Nutr. 2015 Nov 13;6(6):842-51. doi: 10.3945/an.115.008870. PMID: 26567205; PMCID: PMC4642419.

Xu, W., and Han, Z. Cloning and phylogenetic analysis of sid-1-like genes from aphids. *J. Insect Sci.* **8**, 30. (2008). doi: 10.1673/031.008.3001

Yu, BT., Ding, YM. & Mo, JC. Behavioural response of female *Culex pipiens pallens* to common host plant volatiles and synthetic blends. *Parasites Vectors* **8**, 598 (2015). https://doi.org/10.1186/s13071-015-1212-8

Yuan, S., Dahoun, T., Brugarolas, M. *et al.* Computational modeling of the olfactory receptor Olfr73 suggests a molecular basis for low potency of olfactory receptor-activating compounds. *Commun Biol* **2**, 141 (2019). https://doi.org/10.1038/s42003-019-0384-8

Zhou G, Lo E, Githeko AK, Afrane YA, Yan G. Long-lasting microbial larvicides for controlling insecticide resistant and outdoor transmitting vectors: a cost-effective supplement for malaria interventions. Infect Dis Poverty. 2020 Nov 26;9(1):162. doi: 10.1186/s40249-020-00767-3.

Zhu, F., Xu, P., Barbosa, R.R., Choo, Y.M., Leal, W. RNAi-based demonstration of direct link between specific odorant receptors and mosquito oviposition behavior. *Insect Biochem. and Molecular Biol.* **43**(10) 916-923 (2013). https://doi.org/10.1016/j.ibmb.2013.07.008

Zgarbová E, Vrzal R. Skatole: A thin red line between its benefits and toxicity. Biochimie. 2023 208:1-12. doi: 10.1016/j.biochi.2022.12.014.

7 Appendix

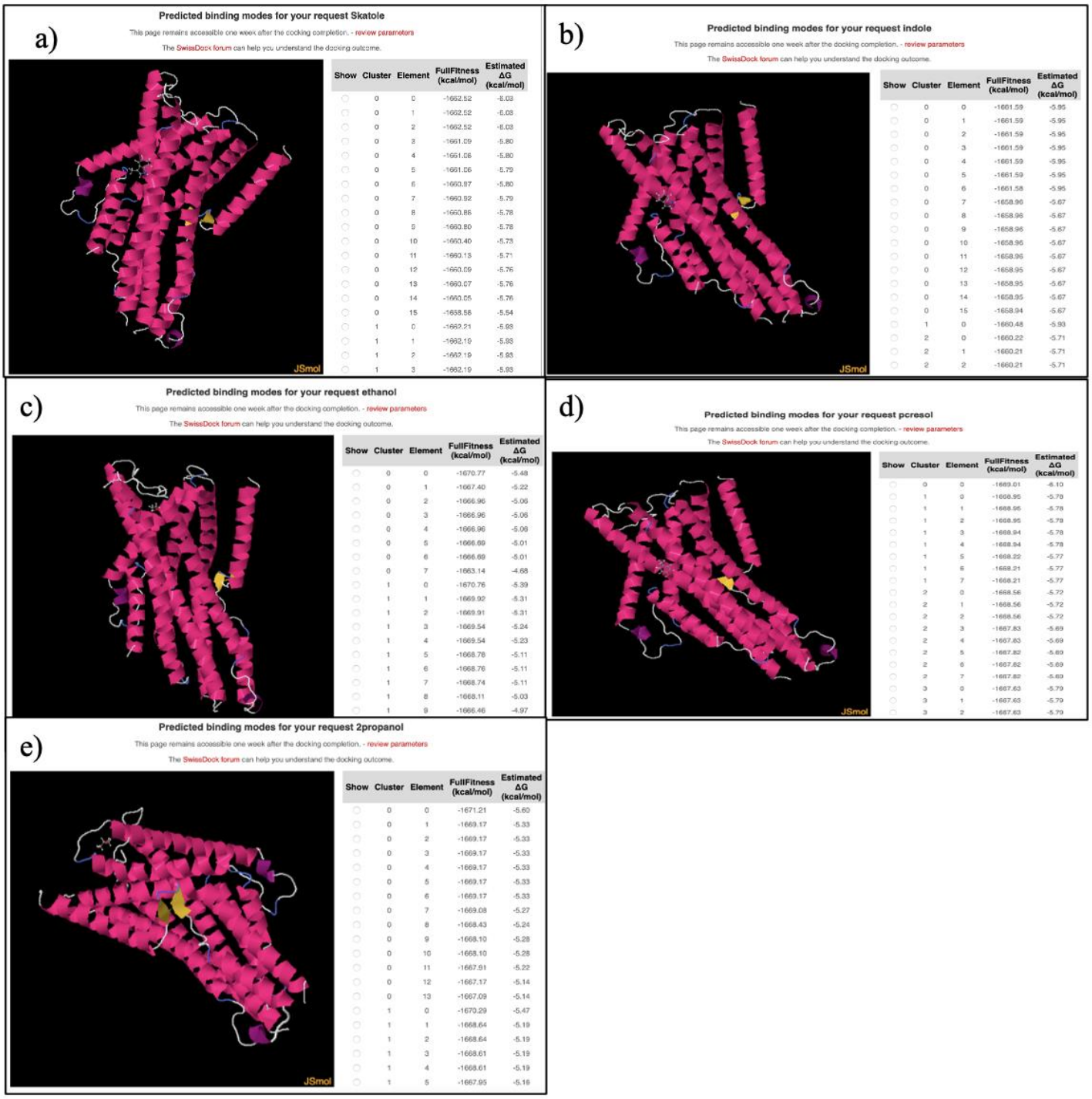
Supplemental Table 1. Odorant Receptors in *Aedes aegypti* with Predicted Homologues in *Aedes albopictus*

	Aedes aegypti	***Aedes albopictus***
	Accession Number:	
OR4	NM_001358193.1	XM_029867968.1
OR2	NM_001358323.1	JN400275.1
OR10	NM_001358339.1	XM_019685388.2
OR34	NM_001358623.1	XM_019674619.2
OR6	NM_001358467.1	XM_019688095.2
OR8	NM_001358457.1	XM_029869708.1
OR31	NM_001358160.1	XM_019698869.2
OR66	NM_001358173.1	XM_019694187.2
OR87	NM_001358336.1	XM_029857535.1
OR7 (ORCO)	NM_001358471.1	XM_029877039.1

Supplemental Table 2. Characterization of the Differences in Extracellular Domains between *Aedes* species

OR targets	Single AA changes *Aae* → *Aal* (:) = size and hydropathy conserved (.) = size or hydropathy are conserved	Extracellular (EC) domains	Insertions or Deletions (Y/N)
OR4	Total = 17 (:) = 5 (.) = 2	Number of EC domains = 4 Number of EC domains with changes = 3	Y (ins *Aal*)
OR7 (ORco)	Identical	Number of EC domains = 4 Number of EC domains with changes = 0	N
OR2	Total = 1 (.) = 1	Number of EC domains = 3 Number of EC domains with changes = 1	N
OR31	Total = 5 (:) = 3	Number of EC domains = 3 Number of EC domains with changes = 1	N
OR6	Total = 36 (:) = 15 (.) = 6	Number of EC domains = 3 Number of EC domains with changes = 3	Y (ins *Aal*)
OR10	Total = 12 (:) = 9 (.) = 1	Number of EC domains = 3 Number of EC domains with changes = 2	N
OR8	Total = 6 (:) = 2 (.) = 2	Number of EC domains = 4 Number of EC domains with changes = 3	N
OR87	Total = 6 (:) = 1	Number of EC domains = 2 Number of EC domains with changes = 2	Y (ins *Aae*)
OR66	Total = 1 (:) = 1	Number of EC domains = 3 Number of EC domains with changes = 1	N
OR34	Total = 34 (:) = 16 (.) = 7	Number of EC domains = 3 Number of EC domains with changes = 2	Y (ins *Aal*)

Supplemental Figure 1. Potential binding of attractive ligands to *Aedes aegypti* OR34. Ligands skatole (a), indole (b), ethanol (c), p-cresol (d), and 2-propanol (e), were docked to the OR34 structure using ConSurf.



Supplemental Figure 2. Angles of the *Aedes aegypti* OR34 protein and *Machilis hrabei* OR5 protein overlayed on top of one another through ChimeraX. Using the predicted structure of *Aedes aegypti* OR34, I-TASSER searched the PDB for predicted matches. The most similar match was 7LICA.