Evaluation of Two dsRNA Delivery Methods into *Aedes aegypti* Larvae to Target Odorant Receptors and the Resulting Behavioural Effects

by

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Abstract

Aedes aegypti are vectors of some of the most serious diseases, contributing to over 300 million cases of infection each year. A key factor underlying their effectiveness as vectors is their ability to detect their host, a process largely mediated by their sense of smell. While mosquito olfaction has been extensively studied in adults, relatively little is known about olfaction in the larval stage. In this study, we investigated *Ae. aegypti* larval olfaction through the characterization of the expression of a few larval-specific odorant receptors (ORs) across development and assessed their potential roles in behaviour. RNA interference was used to knock down three OR genes (OR9, OR34, and OR58) by delivering double-stranded RNA (dsRNA) via two methods: ingestion and absorption. Gene knockdown of OR34 was assessed using qRT-PCR, but no statistically significant reduction in transcript levels was detected. This apparent lack of knockdown is likely due to the very low baseline abundance of these transcripts, the small number of insects treated, and the resulting limited sensitivity to detect any further decrease in expression.

To examine behavioural effects, a chemotaxis assay, modified from a method developed in the Whyard lab, was used to assess larval responses towards the attractive odorants, skatole and indole. A significant reduction in attraction to skatole was observed in larvae after the ingestion of OR9 and OR34 dsRNA. These findings give us a better understanding of the functional roles of ORs in *Ae. aegypti* larvae and provide a foundation for developing control strategies that target larval olfaction. However, further research and optimization are required to improve the detection of odorant receptors in larvae to strengthen and further validate the findings of this study.

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Introduction

The yellow fever mosquito, *Aedes aegypti*, is a major vector of arboviruses, transmitting serious diseases such as Dengue, yellow fever, and Zika virus, which pose significant global health challenges (Patterson et al. 2016). Their ability to detect chemical cues from potential bloodmeal hosts, including humans, makes them highly effective disease vectors. This chemical sensing, known as olfaction, is fundamental to many aspects of mosquito biology. As larvae, the antennae are the main olfactory appendages used to sense predators and locate food (Sih, 1986; Bohbot et al. 2007). As adults, olfactory sensing is carried out by the antennae, proboscis, and maxillary palps. These structures enable them to find nectar sources, and in females, to locate bloodmeal hosts and suitable sites for laying eggs (Bohbot et al. 2007; Khan et al. 2023; Pullmann-Lindsley et al. 2024). The antennae are the primary olfactory appendage and are densely covered with hair-like sensory organs called sensilla (Raji and Potter 2022; Xiong et al. 2025). Sensilla have five different morphologies and function in mechano-, thermo-, hygro-, chemo-reception and olfaction (Ghaninia et al. 2007).

In mosquitoes, there are three classes of olfactory receptors: inotropic receptors (IRs), gustatory receptors (GRs), and odorant receptors (ORs). There are 117 ORs, 135 IRs, and 73 GRs that have been identified in the *Ae. aegypti* genome (Matthews et al. 2018). IRs function in taste, odour, humidity, and temperature detection, GRs primarily function in taste detection, and ORs function in odour detection (Raji and Potter 2022; Fernández-Chiappe et al. 2025; Xiong et al. 2025). The focus of this research is on ORs, which are transmembrane proteins resembling G-protein coupled receptors (Tiwari and Sowdhamini 2023). ORs have seven transmembrane domains, with an intracellular N-

terminus and an extracellular C-terminus (Tiwari and Sowdhamini 2023). Functional ligand-binding ORs create a complex with odorant receptor co-receptors (ORco), forming ligand-gated ion channels (Butterwick et al. 2018; Matthews et al. 2018; Tiwari and Sowdhamini 2023; Fernández-Chiappe et al. 2025). A single OR can detect multiple odorant molecules, and each odorant can activate a unique combination of receptors, further adding to the complexity of mosquito olfaction (Fernández-Chiappe et al. 2025). A particular odorant may activate a certain receptor strongly; alternate odorants may activate this same receptor but to a weaker extent (Fernández-Chiappe et al. 2025). Approximately two to four olfactory receptor neurons are located within each sensillum, with their dendrites housing the odorant's ligand-gated ion channel (Ghaninia et al. 2007; Raji and Potter 2022). Interestingly, a single olfactory neuron can co-express more than one OR, greatly enhancing the mosquito's olfactory abilities (Xiong et al. 2025).

When a mosquito comes into contact with an odorant molecule, the odorant molecule will enter through pores on the antennae sensilla and will migrate into the hydrophilic lymph (the extracellular fluid that circulates in insects) (Xiong et al. 2025). Within the lymph, the odorant molecule will come into contact with odorant-binding proteins (OBPs), whose function is to deliver these molecules to the OR (Xiong et al. 2025). The OBP, with its odorant, binds to the OR complex, resulting in the opening of the ion channel, leading to an influx of cations into the cell, which depolarizes the cell's membrane, resulting in an action potential that activates the neuron to signal to the central nervous system that an odorant has been detected (Raji and Potter 2022). The mosquito then elicits a behaviour change in response to the neuronal activation.

A well-studied odorant-mediated behaviour in adult mosquitoes is host-seeking. During host-seeking, adult female mosquitoes show a typical behavioural response pattern. Once an odorant has been detected, the mosquito will begin flying in its direction (Islam et al. 2017). If the mosquito loses the odorant, it will fly in a side-to-side motion to relocate the source (Islam et al. 2017). The pattern continues until the mosquito is in close range to the host's skin, to which it will then land and feed (Islam et al. 2017). Odorant receptors play a crucial role in discriminating between the vast array of odorant molecules in a mosquito's environment, allowing mosquitoes to detect the location of a specific host. Several odorant receptor genes responsible for host detection in adult *Ae. aegypti* have been characterized. OR2 shows sensitivity towards indole, a major component of human sweat (Bohbot et al. 2011; Ni et al. 2022). OR10 plays a role in the detection of skatole, a component of human odor (Ruel et al. 2019). 1-octen-3-ol, another compound found in human sweat, activates OR8 (Bohbot and Dickens 2009). OR4 plays an important role in helping mosquitoes differentiate between host and non-host animals, mediated by the odorant, sulcatone (McBride et al. 2014). Sulcatone is another component of human body odor and is one of the compounds indicative of a human host (McBride et al. 2014). Other OR genes, such as OR88, OR114, OR115, and OR116, are believed to aid in host-seeking behaviours, although their exact roles are yet to be determined (Ni et al. 2022).

One major focus of mosquito control strategies is disrupting their sense of smell, thereby reducing their ability to locate humans. Repellents are commonly used for this purpose, aiming to limit mosquito-human contact by interfering with the mosquito's natural olfactory responses (Debboun and Strickman 2013). The most widely used and

effective mosquito repellent is N, N-diethyl-3-methylbenzamide, commonly known as DEET (Xu et al. 2019). DEET has been shown to bind to ORs and is thought to disrupt their normal function, confusing the mosquito and preventing it from biting (Pellegrino et al. 2011; Bohbot and Dickens 2012). Bohbot and Dickens (2010) showed that DEET has an effect on both OR2 and OR8 in adult *Ae. aegypti*. DEET on its own resulted in the activation of OR2, and when mixed with other host odors, DEET strongly inhibited OR8 (Bohbot and Dickens 2010). The exact mechanisms of DEET's repellency are still vaguely understood, due to the complexity of mosquito olfaction; other ORs likely play a role in DEET detection. Growing concerns about the potential health effects of DEET have prompted many consumers to seek alternative control methods (Nguyen et al. 2023).

While most current research and control efforts focus on adult mosquito olfaction, relatively little is known about olfactory function in larvae (Kythreoti et al. 2021; Akoton et al. 2024; Fan et al. 2025). Mosquito larvae rely heavily on olfaction to navigate their aquatic environment. *Ae. aegypti* larvae are believed to have a chemo-ortho-kinesis response to stimuli, meaning they change their speed in response to the detection of a stimulus (Lutz et al. 2019, 2020). The behaviour that is altered in response to a stimulus, such as an odorant, is the larvae's swimming speed, to stay within high concentrations of an odorant for an extended period of time (Lutz et al. 2020). Essentially, larvae will swim quickly when they detect an attractive stimulus, and slow down to remain in areas where this stimulus is high in concentration (Lutz et al. 2019). The chemo-ortho-kinesis behaviour elicited by mosquito larvae is thought to be mediated by ORs and the binding of odorant cues found in their aquatic environments to these receptors.

In *Ae. aegypti* larvae, 23 ORs have been identified, 15 of which are specific to the larval stage, based on transcriptomic analyses (Bohbot et al. 2007; Ruel et al. 2019). However, despite this diversity, only a few studies have investigated which chemical ligands interact with these receptors (Mysore et al. 2018; Ruel et al. 2019). The larval-stage specific OR9 has been identified to bind to skatole and indole, both of which are released by bacteria, fungi, and plants, which serve as food sources for the larvae (Ruel et al. 2019). Previous (unpublished) research in the Whyard lab has shown that there are several odorants that are attractive to *Ae. aegypti* larvae, including indole, skatole, ethanol, 2-propanol, and p-cresol. Furthermore, the larval-stage specific OR34 was identified to have potential ligands, including indole, 2-propanol, and ethanol (unpublished data). A study done previously in the Whyard lab showed that these attractive chemicals are no longer attractive to the larvae after RNA interference (RNAi) mediated knockdown of the OR34 gene. The vast array of odorants within a larva's environment indicates their 23 ORs require complex innerworkings to allow them to navigate to food sources and escape predation, suggesting that larval olfaction plays a meaningful role in their behaviour.

Currently, there is very limited knowledge of olfaction in mosquito larvae, with many of the ORs lacking known ligands. A better understanding of olfactory mechanisms in mosquito larvae could open new avenues for mosquito control. While most current control strategies target adult mosquitoes, it may be possible to develop traps that use these compounds as lures. Furthermore, characterizing larval ORs and their ligands could enable the design of molecules that bind these receptors, potentially interfering with the

detection of natural odorants, and thus disrupting essential life processes and providing a novel approach to mosquito management.

This project aims to evaluate RNAi as a tool to silence olfactory genes in mosquito larvae. Specifically, it tests two delivery approaches, direct soaking in double-stranded RNA (dsRNA) solutions and ingestion of bacteria engineered to produce dsRNA, to determine whether these methods can induce gene knockdown and alter larval behavioural responses to odorant cues. Establishing RNAi as a reliable method for targeting larval odorant receptors will be instrumental for future analyses, enabling researchers to pinpoint which odorants bind to specific receptors. Ultimately, this knowledge can guide the design of new compounds that disrupt mosquito olfaction and reduce their ability to locate humans.

The first objective of this study is to assess whether soaking larvae in a dsRNA solution induces RNAi-mediated knockdown of olfactory genes. To target larval-specific ORs, dsRNA of 250 base pairs will be synthesized via *in vitro* transcription. To enhance dsRNA uptake, larvae will be soaked for 2 hours in a solution containing dsRNA, deionized water, and Silwet L-77, a surfactant that may reduce surface tension and facilitate penetration through the larval lipid-coated cuticle. Two days post-treatment, knockdown efficiency will be assessed using qRT-PCR. Four days post-treatment, a chemotaxis assay will be performed to assess whether the knockdown affects larval behavioural responses to odorant stimuli. It is hypothesized that *Aedes aegypti* larvae in a dsRNA solution containing Silwet L-77 will induce sequence-specific RNAi, significantly reducing mRNA levels of the targeted larval odorant receptors at 48 hours

(qRT-PCR) and diminishing chemotactic responses to their cognate odorants at 96 hours, compared to vehicle, non-target dsRNA, and no-surfactant controls.

The second objective of this study is to determine whether ingestion of bacteria expressing dsRNA induces RNAi-mediated knockdown of olfactory genes. Fragments of larval-specific OR genes will be cloned into the pL4440 plasmid and transformed into *Escherichia coli* strain HT115 cells, which are engineered for dsRNA production. Cultured HT115 cells will be heat-killed and mixed with agar to form bacterial pellets containing the target dsRNA. Larvae will be fed these pellets over a two-day period. Knockdown will be assessed using qRT-PCR and behavioural changes will be evaluated four-days post-feeding using the same chemotactic assay described in Objective 1. It is hypothesized that feeding *Aedes aegypti* larvae heat-killed HT115 *E. coli* expressing dsRNA targeting larval odorant receptor genes will induce sequence-specific RNAi, resulting in significantly reduced OR mRNA levels at 48 hours post-feeding (qRT-PCR) and decreased chemotactic responses to cognate odorants at 96 hours, compared to larvae fed HT115 carrying empty vector, non-target dsRNA, or no-bacteria controls.

Materials and Methods

Aedes aegypti maintenance:

Aedes aegypti eggs were hatched in plastic tubs that contained deionized water. Rabbit food pellets were used as nutrition for the larvae. Pupae were transferred to a screened cage held at 28˚C, 60% humidity, and a 16:8 light:dark photoperiod, where they transitioned into adult mosquitoes. The cage contains a constant supply of a 10% sucrose solution and a weekly supply of fresh rat blood to allow for egg production. A container of

water was present, lined with a damp piece of paper towel, to allow females to lay eggs. The egg-covered paper towel was collected weekly and stored until needed.

Target gene selection:

The 15 *Aedes aegypti* ORs identified as being specific to the larval stage (Bohbot et al. 2007) were initially selected as target genes. Gene sequences were retrieved from the National Center for Biotechnology Information (NCBI) database and, when available, from VectorBase (https://vectorbase.org).

Of these 15 larval-specific ORs, only nine of the corresponding genes were successfully amplified from larval cDNA and confirmed by Sanger sequencing (see below for details). Molecular dynamics analyses were performed by Dr. Katie Mitchell-Koch (Department of Chemistry, University of Manitoba) to predict which ORs might bind some known odorants (indole, skatole) that are attractive to mosquitoes (Del Mármol et al. 2021). In brief, she and her team used AlphaFold2 to predict the structure of the ORs, embedded in a lipid membrane composed of POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine), a phospholipid commonly found in biological membranes (Del Mármol et al. 2021). The membrane-bound protein complex was simulated using the web tool CHARMM-GUI, solvating the complex with water, to represent a more natural integral protein structure. The molecular dynamics of the odorant-OR pairing interactions were first simulated using GROMACS to predict the movement and interaction of the protein's amino acids with its environment (water and lipids) and then using AutoDock Vina to simulate the docking of the odorant to the OR protein in this environment (see Figure 1 for an overview of the steps involved). The odorant-OR pairings nominated for further analyses in this study were selected based on those odorants binding to the OR's pocket located in the

transmembrane region formed by the seven-helix bundle of the OR, as described by Del Mármol et al. (2021). Based on these criteria, three ORs, 9, 34, and 58, became the focus for the subsequent experiments.

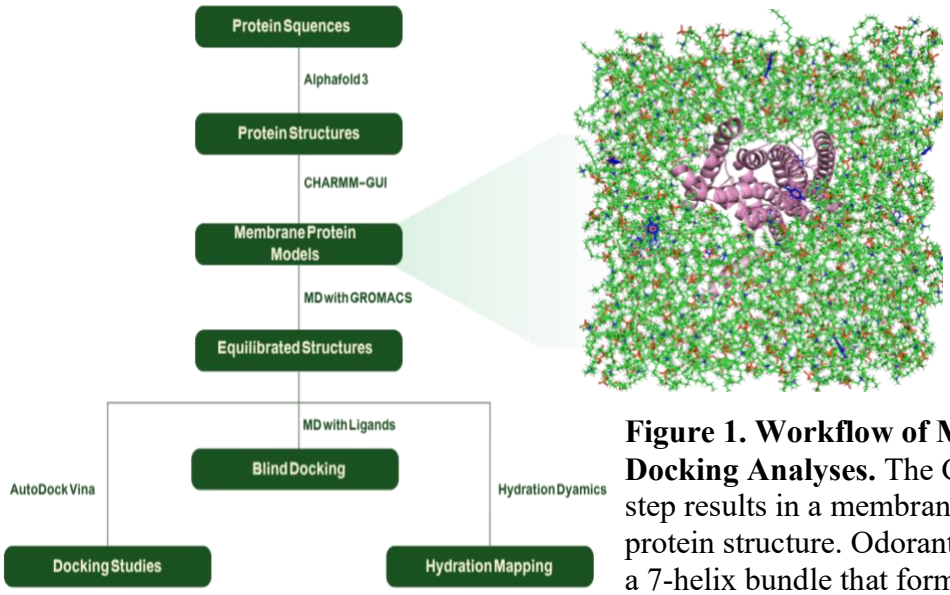


Figure 1. Workflow of Molecular Docking Analyses. The CHARMM-GUI step results in a membrane-embedded protein structure. Odorant receptors have a 7-helix bundle that forms a pore.

Assessing stage-specific expression of OR genes:

To determine the developmental timing of OR gene expression, pools of larvae from each instar stage were collected, ranging from 10 to 30 individuals per pool, depending on larval size (with more first instars and fewer fourth instars). Larvae were homogenized in Lysis buffer (ThermoFisher) containing 2% ß-mercaptoethanol using a Bullet Blender, followed by a QIAShredder (Qiagen) for further disruption. The total RNA was extracted using a ThermoFisher RNA extraction kit. The RNA was treated with TURBO DNase (Life Technologies) to eliminate genomic DNA contamination, then reverse-transcribed into cDNA using the qScript cDNA SuperMix kit (Quanta). Gene-specific primers were designed using Integrated DNA Technologies (IDT) software (Table 1).

Aedes aegypti Gene Name	**Primer Sequence** (F = forward, R = reverse)		**Accession Number**
	For dsRNA	**For qRT-PCR**	
Odorant Receptor 1	**F:**GTATATGGGCAACTGACGATGA **R:**CTCCTTCGAGACTGTGGTTTG		XM_011495003.1
Odorant Receptor 9	**F:**CACATCCCTGGCGTGAATAA **R:**CTCTGATGGTCACTGATGATGG	**F:**GCCTACTTTGCCGTGCTCTA **R:**CCCTGTACACGTCTGCGATT	AAEL006005
Odorant Receptor 14	**F:**GCTGTGACCGGTGTGATAAT **R:**TCCATATGAGTACGCAATCCAC	**F:**GAGGAAACGCAACAGAGAATGT **R:**GCAATGCTTCCTAGTGCGAC	AAEL008442
Odorant Receptor 34	**F:**ACATTGCCTACCTGCTTTCA **R:**CGAAACTGTTCTCTCGATCTCC	**F:**GTAAATTTGCCAGCCTGCCG **R:**CTCTCGATCTCCCGTGCAAA	AAEL003395
Odorant Receptor 37	**F:**AGTTATCAAGTGAGCGGTATG **R:**GTGAATGTCGCAGGAGAGTAG	**F:**ACGCACCAACAAACCAATGA **R:**GAGCGAGTATGCCGAGTTCA	AAEL020435
Odorant Receptor 40	**F:**CTTCAAGTCGGGCTCGTTTA **R:**TAGTCGTTCTTGGTGCCTTG	**F:**ATGGCCACAGTGACTGATCT **R:**GGTTTCGCCCAAATAGCAGA	AAEL005767
Odorant Receptor 48	**F:**CCGTACATGGACCACTTCAA **R:**GCAAGATAGTGGGCTCTAAT	**F:**CCCTGCGCTAACATTCTACAAG **R:**AGTTCATTGGGAAAGTTGAAGTAGA	AAEL020825
Odorant Receptor 58	**F:**GTCAGGCTGGTTCACCTATTT **R:**AGGCTTGTTCGATGGTTGT	**F:**TGCTGAACGGTTTCCTGTGT **R:**AACGCTGGTTGAACGCTTTC	XM_001657514.2
Odorant Receptor 60	**F:**CCTATCGATCTTACCGGCTTAC **R:**CTCGCCATGCAATTCTTTCC		NM_001358794
Odorant Receptor 61	**F:**TGACTGTTCGCCAGAATACC **R:**CGACGACTACTTTCAGCTCTAC		NM_001358791
Odorant Receptor 73	**F:**CGTCCAGCACTTCGTCATTA	**F:**CCGTTTGCGTGGTTTCTGTT	NM_001358130.1

	R:CGGCTAACGAGC ACCATATAA	**R:**AACGATAGCCGAC GGATTCC	
Odorant Receptor 74	**F:**GCCCTTCCTCATC GAGAAATAC **R:**GACCGAAGGTCA TGAACAGAATA		NM_001358779 .1
Odorant Receptor 75	**F:**GCTGGCGATCTTC CTTACTT **R:**CTCGCATTGGGTG ATGTCTAT		NM_001358776 .1
Odorant Receptor 89	**F:**GAACTCTCAGCGC CAATTCT **R:**TTGGATCGCACA CGTTCTAC		NM_001358774 .1
Odorant Receptor 90	**F:**GAAGTCGGATGT ACGAGGTAAC **R:**AGAGAAGGAGAC AATCGCTAATAC		NM_001358773 .1
Ribosomal Protein S7		**F:**CCATTGAACACAA GGTCGACAC **R:**GTAGGGCTCCGGG AATTCGA	XM_001660119 .2

Table 1. Gene-specific primers for dsRNA and qPCR.

Quantitative RT-PCR was used to measure transcript abundance of each OR gene across the different larval stages. Detecting transcripts for all ORs in *Aedes aegypti* larvae proved challenging. Many OR genes' transcripts were initially not detected in any of the larval instars examined. Ensuring that the cDNA was of high quality and sufficiently abundant, indicated by lower CT values for the reference gene, helped in a few instances. To further improve cDNA quality, 50mM $MgCl_2$ was added to the cDNA reaction, which enhanced detection for some targets. However, several ORs remained undetectable, even after this modification, and were excluded from the experiment, as their expression levels are likely too low to detect using this method. Relative transcript abundance was calculated using the ΔΔCT method (Livak and Schmittgen, 2001), with the reference gene, 40S ribosomal protein subunit 7 (S7), used for normalization. A minimum of three biological

replicates (i.e., independent larval pools) were analysed per stage to ensure statistical robustness.

dsRNA template preparation:

Whole bodies of 10 larvae from the developmental stage showing peak expression of each OR were collected. Total RNA was extracted and cDNA prepared as previously described. Primers were designed using IDT software to amplify a 250 bp fragment of each target OR gene (Table 1). PCR amplification was carried out using Econotaq DNA polymerase (Promega). PCR products were visualized by agarose gel electrophoresis, stained with ethidium bromide and observed under an ultraviolet transilluminator. Bands of the expected size were excised and purified using an E.Z.N.A Gel Extraction Kit (Omega). A second round of PCR amplification was performed to increase the yield of purified DNA for downstream ligation reactions.

Purified PCR products were ligated into pJET 1.2 plasmids using a CloneJET PCR Cloning Kit (ThermoFisher). The recombinant plasmids were transformed into *E. coli* DH5-α competent cells, which were plated on Luria-Bertani (LB) agar containing ampicillin (Amp). Colonies were screened by PCR to confirm the presence of the OR insert. Positive colonies were used to inoculate LB + Amp liquid broth and incubated overnight at 37˚C. Plasmid DNA was extracted using an E.Z.N.A. Plasmid DNA Mini Kit (Omega) and sent for sequencing to verify the identity of the insert.

Of the 15 larval-specific OR gene fragments targeted, nine were successfully PCR amplified from larval cDNA and cloned into the pJET 1.2 plasmid (ORs 9, 34, 40, 48, 58, 60, 73, 89, and 90). For three ORs (14, 37, and 61), plasmid transformation of PCR

products into *E. coli DH5-α* competent cells were successful, and PCR screening confirmed inserts of the expected size. However, sequencing revealed that the products were not the correct gene fragments. A second attempt produced similar results, and these ORs were then excluded from the study. Ideally, primer redesign would have been considered to resolve this issue, but time constraints prevented further optimization. ORs 74 and 75 were amplified successfully by PCR, but subsequent plasmid transformation into DH5-α cells failed. Finally, sequencing of OR1 revealed that its sequence was very similar to OR34, differing by an additional stretch of 154 nucleotides at the start of the gene, as well as a 63- and 75-nucleotide region towards the end of the gene. Given this high similarity, and Basic Local Alignment Search Tool (BLAST) analysis confirming closer alignment to OR34, efforts were focused on OR34 rather than treating OR1 as a separate target.

Bacterially-produced dsRNA preparation for larval feeding:

For bacterial delivery of the dsRNA, plasmid DNA from the nine confirmed DH5-α clones and the pL4440 vector were digested using FastDigest restriction enzymes *Xho*I and *Xba*I (ThermoFisher) at a ratio of 1 ng plasmid to 1 µL of enzyme. Digested products were resolved by agarose gel electrophoresis, and the bands corresponding to the digested pL4440 and the OR gene inserts were excised and purified. The OR gene fragments were then ligated into pL4440 using T4 Ligase (ThermoFisher), and the recombinant plasmid was then transformed into HT115 *E. coli* cells. The cells were grown overnight on an LB + Amp agar plate and PCR-screened for the presence of the insert.

To produce bacterial pellets for larval feeding, an overnight culture containing the recombinant pL4440 plasmid was grown in LB + Amp + Tetracycline liquid broth. This culture was added to a baffled flask containing 150 mL of LB broth supplemented with 150

µL of Amp and 150 µL of Tetracycline, incubated at 37˚C with shaking until reaching an optical density (OD_{600}) of 0.6-0.8. To induce dsRNA expression, 650 µL of 100 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added, and the culture was incubated for an additional 4 hours. Following induction, 50 mL aliquots of the culture were centrifuged at 3650 x g for 5 minutes to pellet the bacteria. The LB was decanted, and 300 mg Brewer's yeast in 3 mL deionized water was added, followed by 12 mL molten 1% agarose. Next, the bacteria were heat-killed in a water bath at 75ºC for 10 minutes. The liquid was then poured into syringe barrels and stored at 4ºC.

In vitro transcribed dsRNA for larval soaking:

To prepare dsRNA for larval dsRNA soaking experiments, the gene-specific primers used to create the dsRNA (Table 1) had a 17-nucleotide T7 promoter sequence added to amplify a 250 bp fragment of each OR gene. PCR amplification was performed to generate the transcription template, and products were resolved on an agarose gel to confirm the presence of the expected band containing the T7 promoter and gene of interest. The PCR product, either directly or following gel-extraction, was purified and used as a DNA template for *in vitro* transcription. The dsRNA was synthesized using the MEGAscript® RNAi Kit (ThermoFisher), following the manufacturer's protocol.

dsRNA delivery:

To induce RNAi knockdown in the mosquito larvae, the previously prepared dsRNA was delivered to the larvae using two methods. In both approaches, a β-glucuronidase (GUS) gene from *E. coli* was used as a negative control to confirm that the species-specific dsRNA is responsible for any observed effects in the mosquito larvae.

For the soaking method, a solution was prepared using the *in vitro* transcribed dsRNA at a concentration of 250 ng/µL, 0.0125% Silwet L-77 (Fisher) and deionized water. One-hundred-twenty 3rd instar larvae were placed in 670 µL of the dsRNA solution for 2 hours. After soaking, the larvae were evenly distributed into three petri dishes containing 75 mL of deionized water and a rabbit food pellet for nutrition, resulting in three biological replicates per treatment. Ten larvae were collected from each petri dish 2 days post-treatment, and another ten were collected at 4 days post-treatment. Treatments for a single assay included: the OR34 gene dsRNA; the OR34 gene dsRNA with Silwet; GUS gene dsRNA; GUS gene dsRNA with Silwet; Silwet only; and water only.

To assess the effects of dsRNA ingestion, bacterial pellets prepared earlier were used. Two 0.5 mL slices of the bacterial pellet were placed into a petri dish containing 75 mL of deionized water. Fifty larvae were added to each dish, and new pellets were added once the previous ones were consumed. Larvae were collected using the same sampling times as the soaking assay. Three biological replicates were conducted for each treatment, which included: a bacterial pellet with the OR gene dsRNA; a bacterial pellet with the GUS gene dsRNA, and a rabbit food pellet. The OR genes used include OR9, OR34, and OR58.

Assessing knockdown using Quantitative Reverse Transcriptase PCR Analysis:

To assess knockdown of the OR gene of interest, qRT-PCR was performed on the ten larvae collected from both treatments after 2 days. Primers were created using Primer 3 that span intron regions, when possible, in the OR gene of interest (Table 1). *Aedes aegypti* 40s ribosomal protein S7 was used as a reference gene to normalize the amount of cDNA in each sample. The BioRad CFX Connect Real-Time PCR System was used with SsoFast EvaGreen Supermix (BioRad). As previously discussed, detecting the OR transcripts was

challenging, likely due to their low abundance within the organism. The solutions outlined previously were applied to address this issue. Despite these optimizations, multiple qRT-PCR attempts were conducted, and cDNA quality remained critical for successful OR transcript detection. Detecting knockdown was also difficult, as several qRT-PCR runs were needed to generate values for calculating relative transcript abundance in both the control and treated samples. Consequently, the focus was limited to ORs 9, 34, and 58. Relative transcript abundance was calculated using the ΔΔCT method (Livak and Schmittgen 2001). Samples were removed if the Ct values for the reference gene were greater than 30, as this signifies low cDNA abundance and unreliable results.

Chemotaxis assay:

To assess whether the knockdown of larval-specific OR genes of interest results in an observable behavioural response in the mosquito larvae, a chemotaxis assay was performed four days after the larvae were treated with dsRNA as previously described. The assay technique in this study was previously established in the Whyard Lab (unpublished data), which was modelled after a larval mobility assay developed by Dr. Laurence Zweibel and described in Liu et al. (2023). An arena was created using a uniform 27 cm long rectangular container, marked into 3 equal



Figure 2. Chemotaxis Assay Set-up. The arena used in the chemotaxis assay is one of the troughs; the filter paper discs were added to the top and bottom sections, indicated by the stars. The 3 replicates are indicated by R1-R3.

sections (Figure 2). For each assay, the arena was filled with 300 mL of deionized water, and a 1 cm circular filter paper disc was placed on both sides of the arena. A small volume (3µL) of the odorant at a concentration of 0.01 mM, dissolved in Dimethyl sulfoxide (DMSO), was applied to the filter paper, which was then placed into the arena 1 minute before the larvae were added. For each dsRNA treatment, an initial assay was run using 3µL of 0.01 mM DMSO as the "no chemical control", to compare to the odorant/chemical (skatole or indole) assays. The position of the attractant and negative control filter paper was switched in each trial to ensure no bias towards one end of the arena (Table 2).

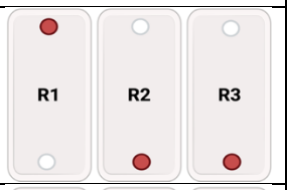
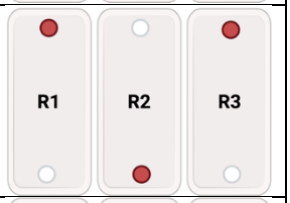
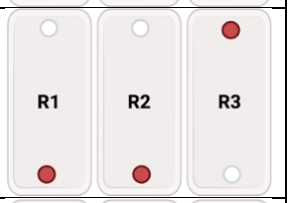
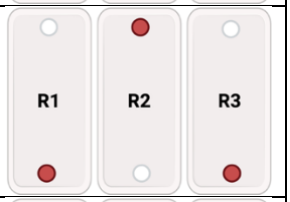
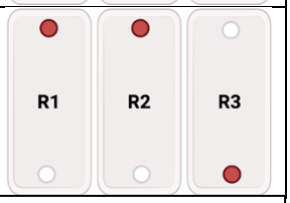
Treatment	Arena Set-up
Food Pellets	
GUS dsRNA	
OR9 dsRNA	
OR34 dsRNA	
OR58 dsRNA	

Table 2. The Position of Attractant in the Chemotaxis Assay. The filter paper configuration for the chemotaxis assay performed on the larvae post-treatment. The white circles indicate the plain filter paper (negative control); the red circles indicate the filter paper with the odorant/chemical. The three replicate arenas are labelled R1 to R3.

The larvae, in groups of 10, were placed into the centre of the arena after being starved for 3 hours, with 3 replicates being run simultaneously. A camera was set up above the arenas to record the positions of the larvae during the entire 10-minute assay. Snapshots of the video were taken at timepoints 5, 7.5, and 10 minutes to compare the behavioural responses exhibited by the larvae. The use of video proved to be useful to evaluate when the negative control, dsRNA-GUS-treated larvae,

were most evenly distributed. Based on these videos, the 5-minute time point was selected for all subsequent analyses, as at this time, the negative control larvae had dispersed from their starting positions and were the most evenly distributed along the length of the arenas, and thus could serve as a useful comparison to the other treatments (Appendix A).

The larvae's responses were measured using a binomial system where larvae found within the 1/3 of the arena closest to the odorant were scored as a positive chemotactic response (=1), and those in the other 2/3 of the arena were scored as a no chemotactic response (=0). The odorants, skatole and indole, and their concentrations selected for this study were those found to be attractive based on the research conducted by a previous student in the Whyard lab.

Statistical analysis:

Statistical analyses were performed using RStudio (version 2026.01.1+403), and a p-value ≤ 0.05 was considered significant. The statistical significance of OR expression between instars was calculated using a one-way Analysis of Variance (ANOVA) test. A one-way ANOVA test is used to ensure the difference in expression between the instars is not due to random chance. A post-hoc pairwise comparison, Tukey HSD, was used as a follow-up to correct for any false positives produced by the ANOVA test and to detect any significant differences between the individual groups examined. A Mann-Whitney U test was used to assess the knockdown in the Quantitative Reverse Transcriptase PCR analysis. A non-parametric test is required as the sample size was too small to claim that the data follows a normal distribution. A two-portion z-test was used to determine statistical significance in the chemotaxis assay. The goal was to determine if there was statistical

significance between the proportion of larvae attracted to the odorant in the GUS dsRNA-treated larvae compared to the OR dsRNA-treated larvae.

Results

Stage-specific expression of OR genes:

The expression of OR9 and OR34 across different larval stages was evaluated using qRT-PCR. While all larval instars showed some expression of these genes, their levels varied during development. OR9 transcripts were more abundant in the first and second instars, but decreased by approximately 50 and 40% in the third and fourth instars, respectively (Figure 4). A one-way ANOVA showed that the decrease observed in the 3rd and 4th instars corresponded to a statistically significant difference in mean OR9 expression across instars (P-value = 0.034). However, the post-hoc pairwise comparison (Tukey HSD) did not identify any significant pairwise differences between the individual instar groups.

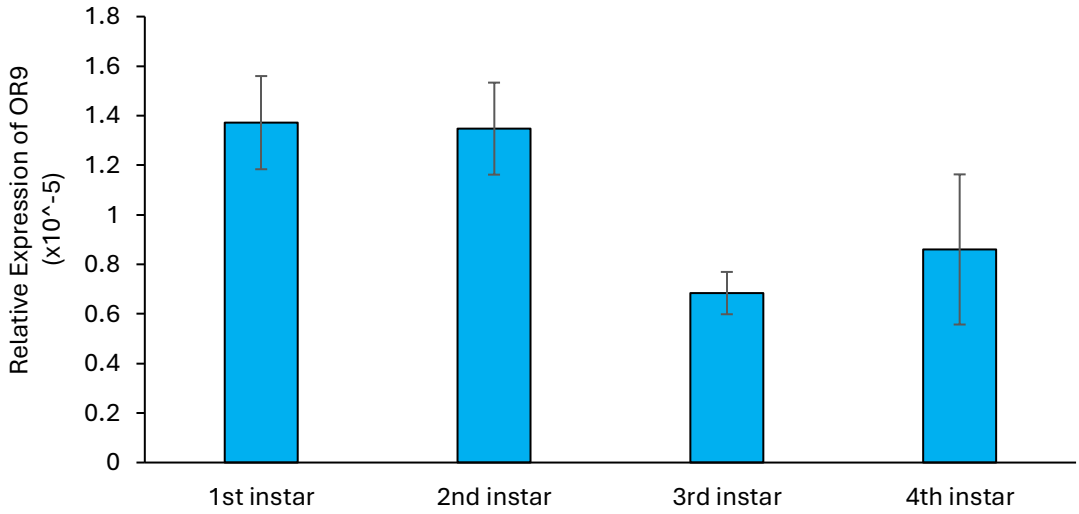


Figure 3. Expression During Larval Development of OR9 Relative to Ribosomal Protein S7. The values represent the means and standard errors of three replicate experiments: 30, 20, 15, and 10 pooled larvae from 1st, 2nd, 3rd, and 4th instars, respectively, were used per replicate. A one-way ANOVA revealed a statistically significant difference in mean OR9 expression between instars ($p = 0.034$); however, post-hoc Tukey HSD analysis did not indicate any significant pairwise differences ($p > 0.05$).

In contrast, OR34 transcripts were about 2-fold more abundant than OR9 transcripts in the first and second instars, and their levels increased in the third and fourth instars, reaching an abundance 8-fold greater than OR9 by the fourth instar (Figure 5). The differing expression levels of OR34 among the 4 instars were not significant when examined using a one-way ANOVA ($p = 0.053$). Additionally, a Tukey HSD pairwise comparison test determined that there were no significant differences between the instar groups.

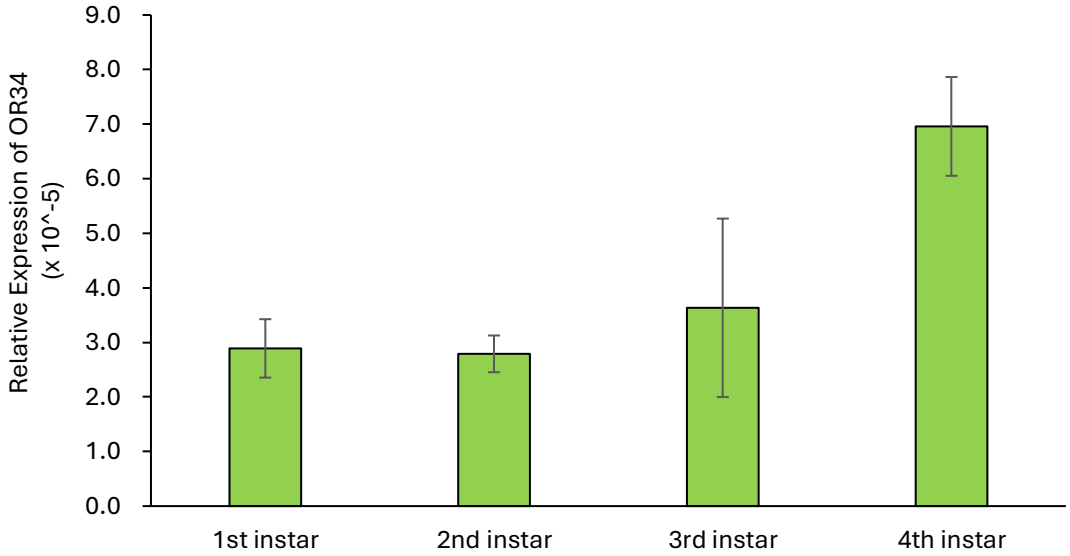


Figure 4. Expression During Larval Development of OR34 Relative to Ribosomal Protein S7. The values represent the means and standard errors of three replicate experiments: 30, 20, 15, and 10 pooled larvae from 1st, 2nd, 3rd, and 4th instars, respectively, were used per replicate. There were no statistically significant differences in OR34 expression levels between instars, as determined by a one-way ANOVA followed by a post-hoc Tukey HSD test ($p > 0.05$).

Overall, the relative expression levels of the OR genes were very low. Due to the difficulty in detecting OR transcripts and time constraints, the analysis of stage-specific expression for the other OR genes was not possible. Since the results of the developmental profiling did not identify any instar with a largely greater abundance of an OR transcript, $3^{rd}/4^{th}$ instar larvae were used in the two-dsRNA treatments. The $3^{rd}/4^{th}$ instar larvae had the overall highest relative expression of an OR gene, OR34, and the use of a single instar reduced the number of controls required.

Protein docking analysis:

To predict the binding of known attractive odorants to ORs, molecular dynamics analyses were performed by Dr. Mitchell-Koch and her team. They analyzed images created by Autodock Vina of the binding of a variety of odorant molecules to ORs. An odorant that binds into the transmembrane core of the 7 alpha helices is likely to indicate the specific affinity of the OR towards this ligand (Del Mármol et al. 2021). Dr. Mitchell-Koch relied on the odorant-docking imaging and determined that OR34 is likely to bind to skatole and OR58 to indole (Figure 5). The images created show the close relationship the odorants have with the transmembrane core of the 7 alpha helices of the ORs of interest. These findings added to the evidence that formed the decision to focus on skatole and indole as the attractive odorants used in the chemotaxis assay.

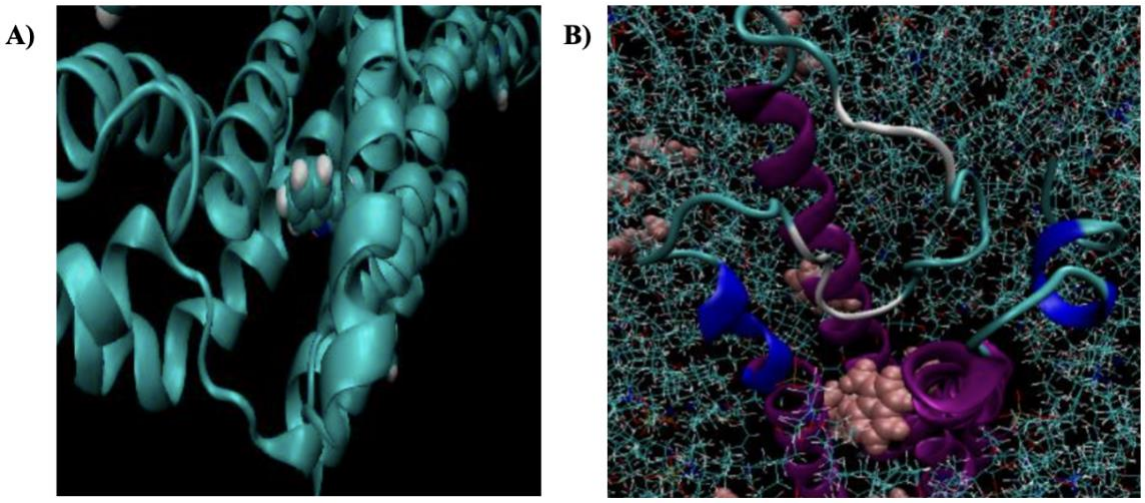


Figure 5. Simulation of Odorants Docking to Odorant Receptor. Autodock Vina images of the binding of two odorants to two ORs, generated by Alpha-fold2. **A)** OR58 is viewed from the side, without the lipid bilayer present, and indole (blue and white) is situated with the 7 transmembrane alpha helices. **B)** OR34, viewed from above, with membrane lipids surrounding the protein and skatole (pink) embedded into the transmembrane core of the protein, with alpha helices coloured blue and purple.

OR knockdown analysis:

To assess the knockdown efficiency for the two dsRNA delivery methods, a qRT-PCR analysis was used to quantify the expression of OR genes relative to the reference gene, ribosomal protein S7. Despite extensive optimization of cDNA synthesis and qRT-PCR conditions, OR9 and OR58 transcripts remained inconsistently detectable. Because their expression could not be measured reliably, these genes were excluded from knockdown analysis. However, the transcript abundance of OR34 was measurable in the dsRNA-treated larvae, and a 35% reduction was observed in larvae that had ingested bacteria expressing OR34 dsRNA, relative to the negative control, although this difference was not statistically significant (Figure 6; Mann-Whitney U test, $p = 0.70$). One replicate was removed from the dsGUS-treated samples as the Ct value for the reference gene was above 30 (~31), indicating a poor RNA yield and the OR34 was consequently not detectable.

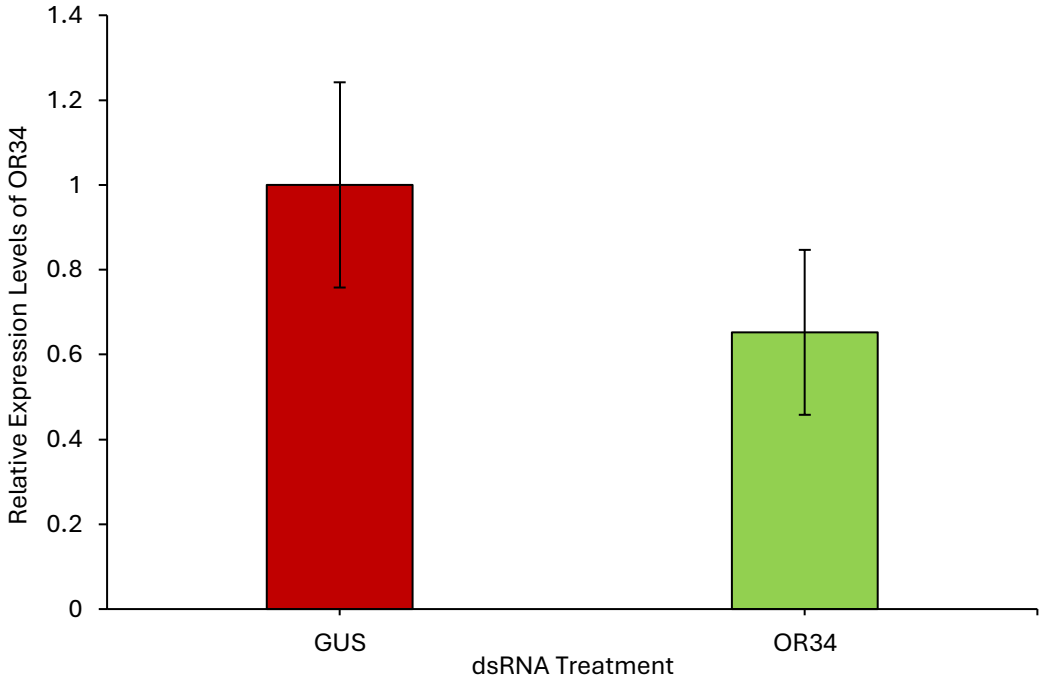


Figure 6. OR34 Knockdown: Ingestion of dsGUS vs. dsOR34. The expression levels of OR34 were detected using qRT-PCR relative to the reference gene, ribosomal protein S7. Ten 4th instar larvae were collected after ingesting bacterial pellets containing dsRNA of OR34 and GUS for 2 days. The values represent the means and standard errors of ten larvae pooled, with a total of three replicates for the dsOR34 treatment and two replicates for the dsGUS treatment. There was no statistically significant difference between OR34 expression levels in the dsGUS control compared to the dsOR34 treatment, observed using a Mann-Whitney U test ($p > 0.05$).

There was an increase in the amount of relative OR34 expression in the larvae soaked in OR34 dsRNA (without Silwet) compared to the GUS dsRNA control. The relative expression levels of OR34 had three times the expression in the treated sample compared to the GUS control; however, this difference was not statistically significant due to the small sample size (Figure 7; Mann-Whitney U test, $p = 0.67$). Once again, one replicate was excluded from both the control and treated samples as the Ct values for the reference gene were greater than 30 (~34-35), reflecting a low yield of RNA and undetectable levels of OR34 transcripts.

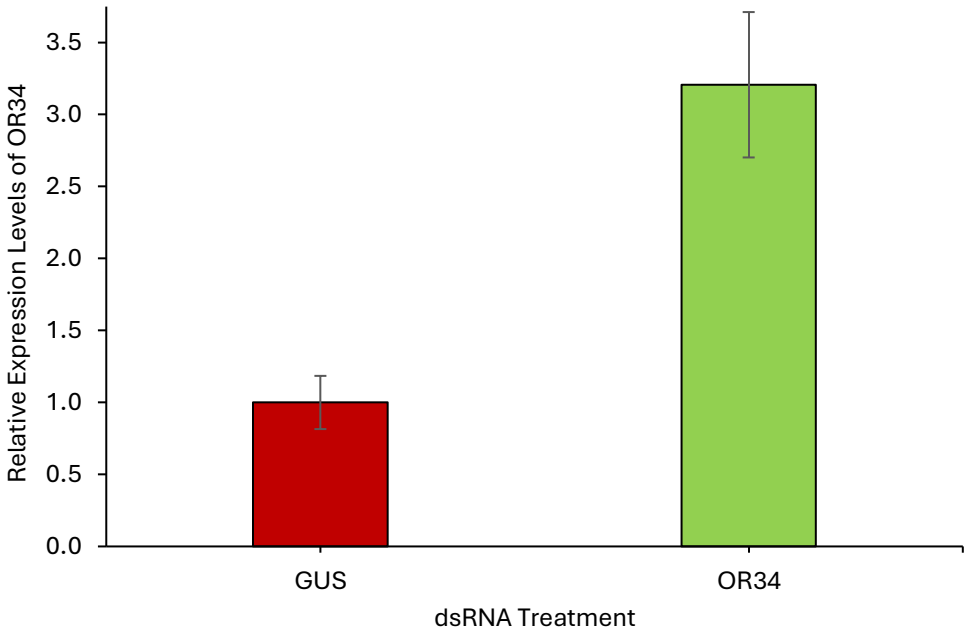


Figure 7. OR34 Knockdown: Soaking in dsGUS vs. dsOR34. The expression levels of OR34 relative to the reference gene, ribosomal protein S7, were detected using qRT-PCR. Ten 4th instar larvae were collected 2-day post treatment, where they were soaked in a solution containing GUS and OR34 dsRNA. The values represent the means and standard errors of ten pooled larvae, with a total of two replicates. There was no statistically significant difference between the OR34 relative expression levels in the dsGUS control compared to the treatment (Mann-Whitney U; p-value > 0.05).

The larvae soaked in dsRNA and Silwet had a knockdown similar to the larvae treated with bacterial pellets containing OR34 dsRNA. The larvae treated in the solution containing dsRNA and the surfactant Silwet had a 36% reduction in the relative expression of OR34 in the treated samples compared to the control, although this knockdown was not statistically significant (Figure 8; Mann-Whitney U test, $p = 0.33$). One replicate was excluded from both the control, due to a double peak in the melt curve, and the treated samples, due to the absence of OR34 detection, resulting in only two replicate measures.

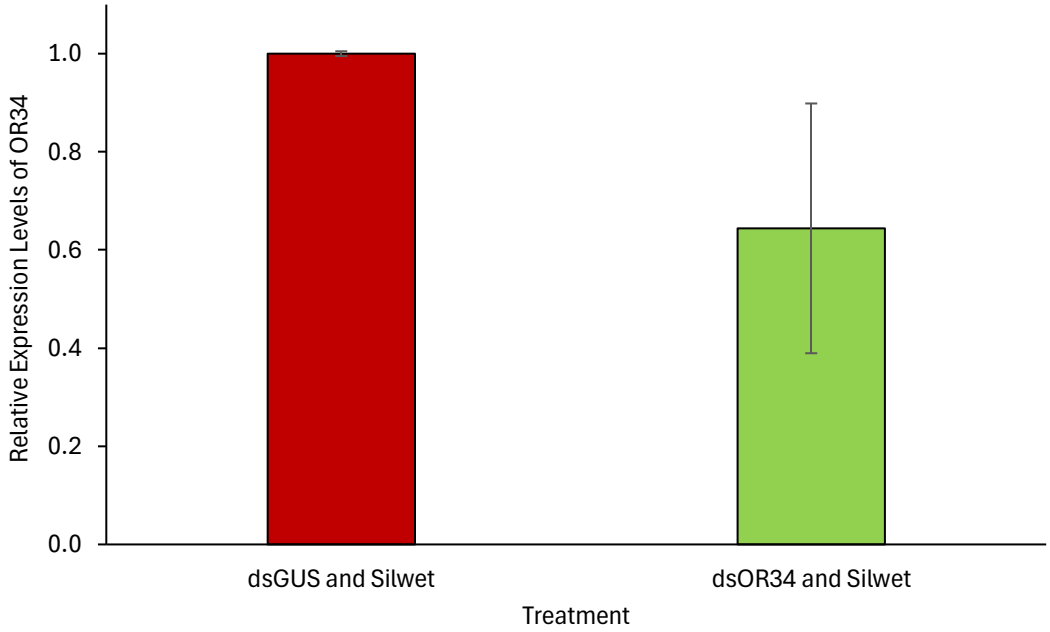


Figure 8. OR34 Knockdown: Soaking in dsGUS vs. dsOR34 and the surfactant Silwet. The expression levels of OR34 were measured using qRT-PCR, relative to the reference gene, ribosomal protein S7. The values represent the means and standard errors of two replicates of ten pooled larvae. Ten 4th instar larvae were treated in a solution containing dsRNA targeting the genes GUS and OR34 with the addition of the surfactant Silwet, 2 days before being collected. There was no statistically significant difference in the relative expression levels of OR34 between control, dsGUS, and the treated samples, dsOR34, using the Mann-Whitney U ($p > 0.05$).

Chemotaxis assay analysis – ingested bacterial dsRNA delivery:

A chemotaxis assay was conducted using mosquito larvae 4 days post-dsRNA treatment. The movement toward two attractive odorants, indole and skatole, was assessed in the larvae that ingested bacterial pellets containing dsRNA targeting 3 OR genes (9, 34, and 58). In the no chemical control, a response of 0.33, or 10/30 larvae, would represent a random distribution of larvae, as the "proportion of larvae attracted" refers to the proportion of larvae in the 1/3 of the tray closest to the odorant source. In the no chemical controls (i.e. where DMSO was used in place of the attractant), the proportion of larvae attracted ranged from 0.40-0.43 for the feeding assays, acting as the baseline for the ensuing experiments.

The distribution of larvae in the no odorant control assays showed no difference between the GUS- and OR9-treated larvae in their locations within the arena, indicating that the knockdown of OR9 did not impact general locomotion of the larvae (Figure 9; two-proportion z-test $p = 0.793$). However, there was a 46% reduction in the proportion of larvae attracted to skatole, which was considered statistically significant using a two-proportion z-test ($p = 0.037$). Additionally, there was a 50% reduction in the proportion of larvae attracted to indole, but this trend toward reduced chemotaxis was not statistically significant (two-proportion z-test $p = 0.091$).

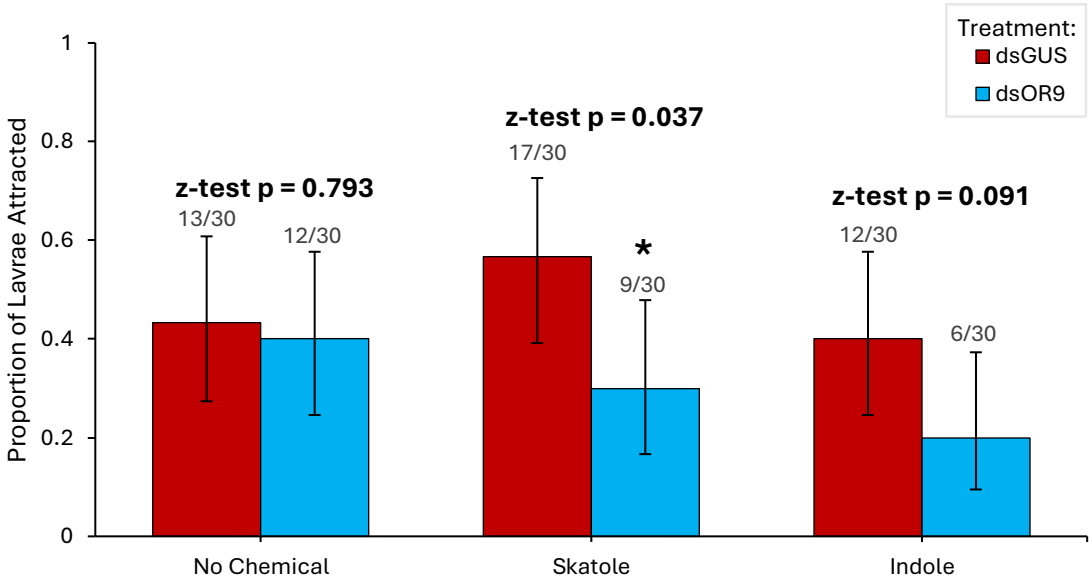


Figure 9. Mosquito Larvae Chemotaxis: Ingestion of dsGUS vs. dsOR9. *Ae. aegypti* larvae's behavioral response to the attractive odorants, skatole and indole. To induce RNAi-mediated gene knockdown, mosquitoes were fed bacterial pellets containing dsRNA of the genes OR9 and GUS (control) for 4 days. The values represent the proportion of larvae attracted with the 95% Wilson confidence intervals depicted by the vertical lines. Thirty 4th instar larvae were used per treatment. The fractions indicate the number of larvae attracted over the total number of larvae. A statistically significant decrease in attraction to skatole was observed (two-proportion z-test, $p = 0.037$), but the reduced attraction to indole was not statistically significant ($p = 0.158$).

The feeding of bacterial pellets containing OR58 dsRNA resulted in very little change in the larvae's movements (Figure 10). In the no odorant control, there was no difference in the distribution of the GUS- and OR58-treated larvae within the arena, again indicating that the OR-specific dsRNA had no impact on larval movements (two-proportion z-test $p = 1.00$). In the skatole assay, there was a 23% reduction in positive chemotaxis to the odorant in the OR58-treated larvae, relative to the GUS-treated larvae, but this decrease was not statistically significant (two-proportion z-test $p = 0.302$). The larvae treated with OR58 dsRNA had a 25% increase in the proportion of larvae attracted to indole, which was not statistically different from the negative control (two-proportion z-test $p = 0.436$).

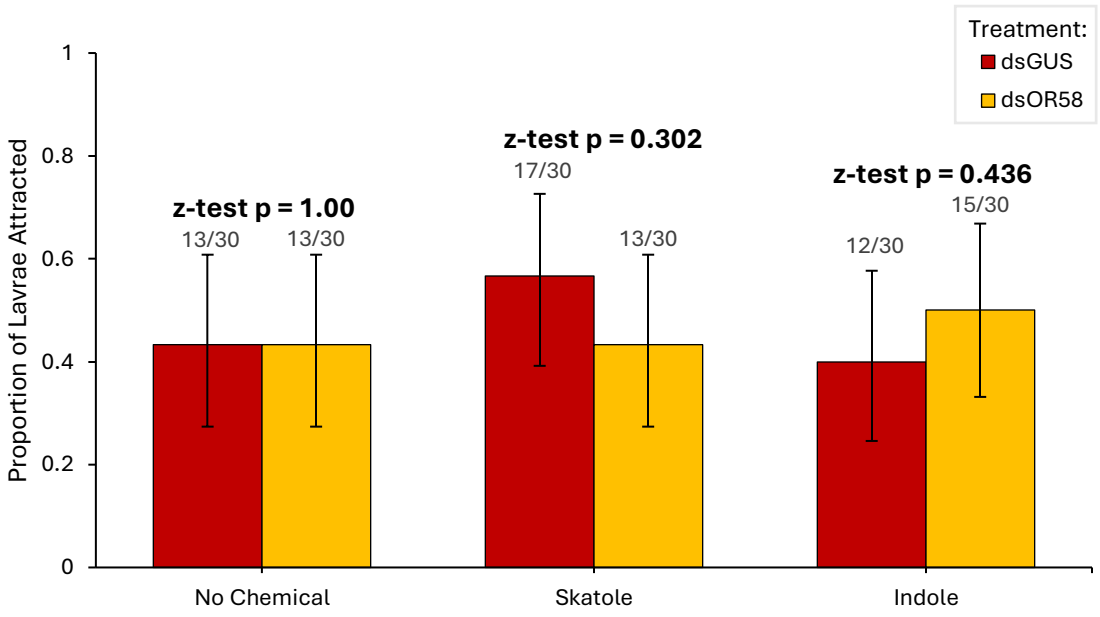


Figure 10. Mosquito Larvae Chemotaxis: Ingestion of dsGUS vs. dsOR58. RNAi-mediated gene knockdown was facilitated through the ingestion of bacterial pellets containing dsRNA of the genes OR58 and GUS (control) for 4 days. The values represent the proportion of *Ae. aegypti* larvae showing positive chemotaxis towards skatole and indole, with a 95% Wilson confidence interval, using 30 insects. The fractions indicate the number of larvae attracted over the total number of larvae. No statistically significant differences were observed between the GUS-dsRNA and OR58-dsRNA treated larvae using a two-proportion z-test ($p > 0.05$).

The larvae's behavior was significantly different after feeding bacterial pellets containing OR34 dsRNA (Figure 11). As before, in the no chemical control, there was no difference in the responses elicited by larvae treated with GUS compared to OR34 (two-proportion z-test $p = 0.793$). There was a significant 59% decrease in the proportion of larvae attracted to skatole in the larvae treated with OR34 dsRNA compared to those treated with GUS dsRNA (two-proportion z-test $p = 0.008$). There was also a modest 42% decrease in the proportion of OR34-dsRNA-treated larvae attracted to indole, relative to the negative controls, although this was not statistically significant (two-proportion z-test $p = 0.165$). Due to the significant reduction in the mosquito larvae's response to skatole after ingestion of dsRNA targeting OR34, this became the focus for the subsequent chemotaxis assays, using the soaking delivery method.

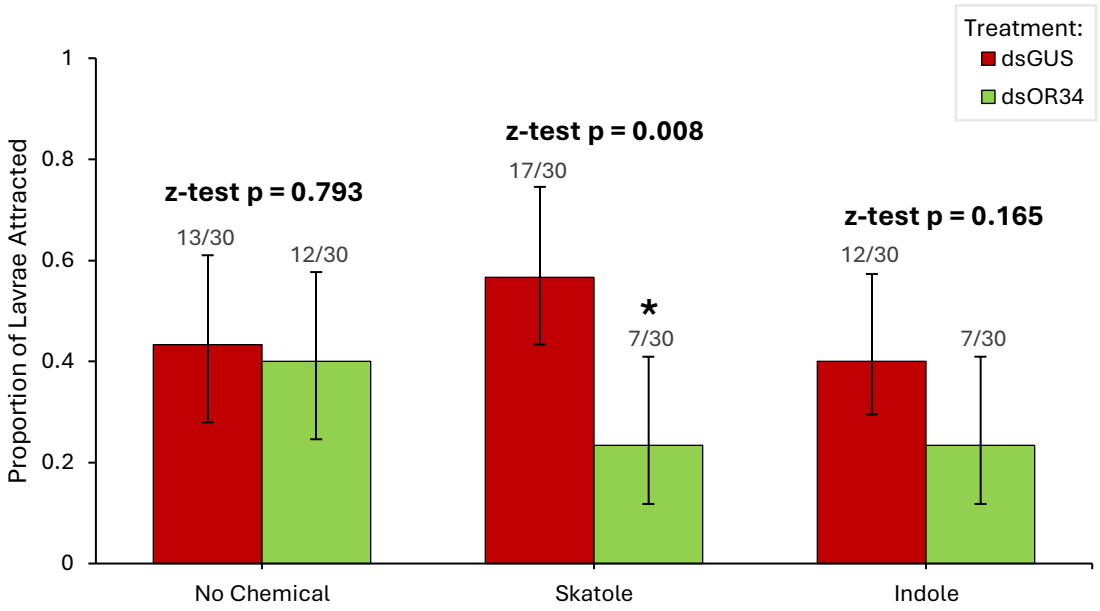


Figure 11. Mosquito Larvae Chemotaxis: Ingestion of dsGUS vs. dsOR34. RNAi-mediated gene knockdown was facilitated through the ingestion of bacterial pellets containing dsRNA of the genes OR34 and GUS (control) for 4 days. The values represent the proportion of *Ae. aegypti* larvae showing positive chemotaxis towards skatole and indole, with a 95% Wilson confidence interval, using 30 insects. The fractions indicate the number of larvae attracted over the total number of larvae. The asterisk indicates a statistical difference between the GUS- vs OR34-dsRNA treatments, determined using a two-proportion z-test (p-value ≤ 0.05).

Chemotaxis assay analysis –dsRNA soaking delivery:

A chemotaxis assay was then performed using larvae treated with OR34 dsRNA using two soaking methods. In the first method, larvae were soaked in deionized water containing OR34 dsRNA. Larvae treated this way showed no significant difference in the proportion attracted to skatole compared to the GUS dsRNA control (two-proportion z-test $p = 1.00$; Figure 12). Moreover, the distribution of larvae in the no odorant control did not differ in their distribution within the arena from the skatole treatment, indicating that neither the dsGUS nor the dsOR34-treated larvae responded positively to the attractant. The lack of a positive response to skatole in the negative controls was unexpected; this might be explained by the fact that the skatole used in this assay was diluted in DMSO to a concentration of 1 M and stored at 4˚C in a dark container for a few weeks prior to this assay. It is possible that the skatole had degraded during its prolonged storage. Due to time constraints, the assay could not be repeated with freshly prepared skatole.

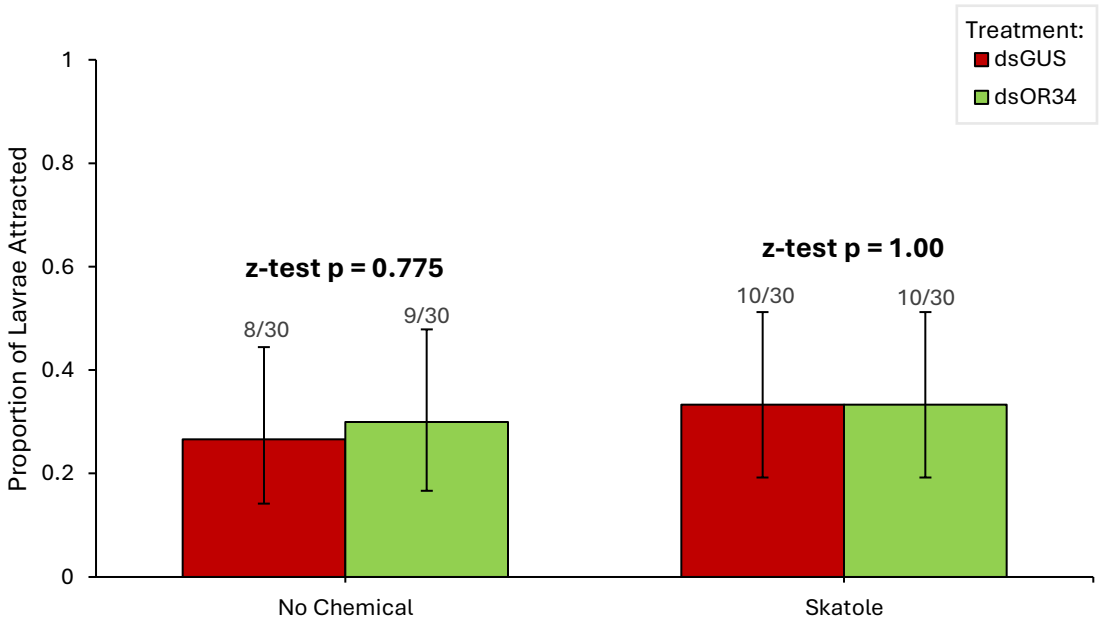


Figure 12. Mosquito Larvae Chemotaxis: Soaking in dsGUS vs. dsOR34. RNAi-mediated gene knockdown was facilitated through the soaking of *Ae. aegypti* larvae in a solution containing dsRNA of the genes OR34 and GUS (control) for 2 hours, 4 days before this assay. The values represent the proportion of larvae showing positive chemotaxis towards skatole, with a 95% Wilson confidence interval. Three experimental replicates, with ten 4th instar larvae pooled per replicate. The fractions indicate the number of larvae attracted over the total number of larvae. No statistically significant differences in the larvae's distributions were observed in these treatments (two-proportion z-test; p-value > 0.05).

The second soaking method exposed larvae to OR34 and GUS dsRNA in deionized water containing 0.0125% Silwet. Notably, adding Silwet altered larval movements not seen in the bacterial dsRNA trials (Figure 13). In the no-chemical control, the proportion of larvae in the "attraction" zone was curiously higher in the dsOR34 group than in the dsGUS group, suggesting the larvae were moving non-randomly, unlike previous treatments. This difference was determined to be statistically significant (two-proportion z-test $p = 0.039$). A similar trend was observed in the skatole assay, where dsOR34-treated larvae again showed a slightly higher proportion in the attracted zone than dsGUS-treated larvae, but the difference was not statistically significant (two-proportion z-test $p = 0.284$).

The addition of Silwet appears to have contributed to these unexpected behavioral patterns. Larvae exposed to 0.0125% Silwet exhibited noticeably slower and abnormal movement four days after treatment. Curiously, instead of distributing randomly in the arena, as seen in earlier assays, OR34 dsRNA-treated larvae were slightly attracted to the "no chemical" control region of the arena, which in fact, was not chemical-free, but contained the solubilizing compound, DMSO. This raises the possibility that Silwet treatment, combined with OR34 knockdown, may have increased larval responsiveness to the DMSO solvent itself, potentially masking or overriding any response to skatole.

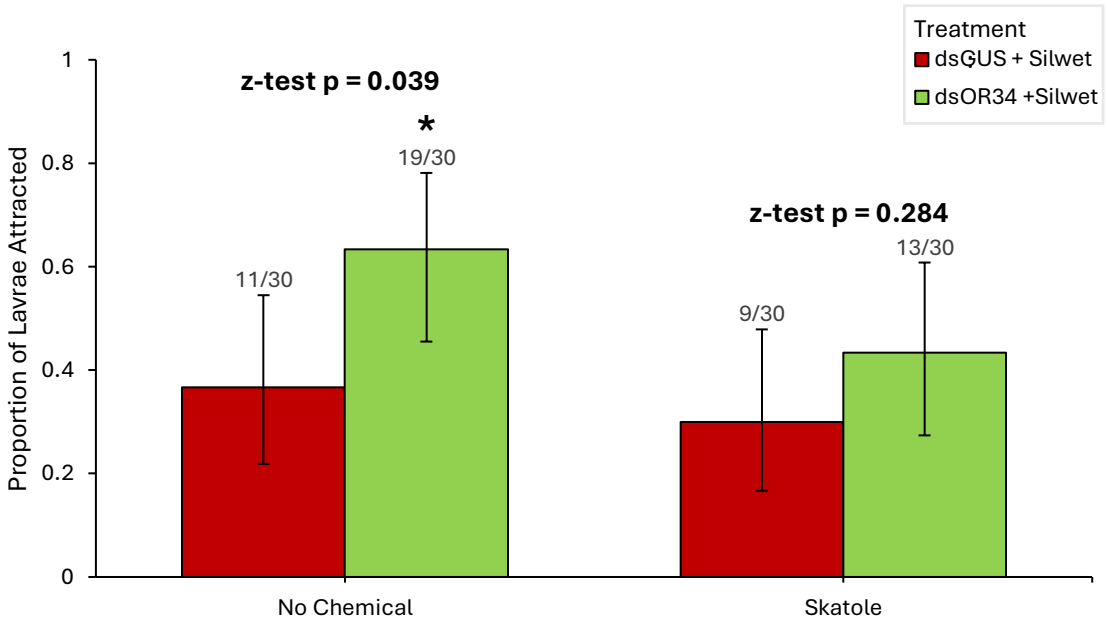


Figure 13. Mosquito Larvae Chemotaxis: Soaking in dsGUS vs. dsOR34 with Silwet. RNAi-mediated gene knockdown was facilitated through the soaking *Ae. aegypti* larvae in a solution containing dsRNA of the genes OR34 and GUS (control) and the surfactant, Silwet, for 2 hours, 4 days before this assay. The values represent the proportion of larvae showing positive chemotaxis towards skatole, with a 95% Wilson confidence interval. Three experimental replicates, with ten 4th instar larvae pooled per replicate. The fractions indicate the number of larvae attracted over the total number of larvae. The increased movement to the attraction zone was statistically significant in the no chemical control, indicated by an asterisk (two-proportion z-test, p-value ≤ 0.05).

Discussion

Despite the importance of olfaction in mosquito larval behaviour, relatively little research has been conducted on how ORs are expressed in *Ae. aegypti* larvae or how these receptors respond to specific odorants. The goal of this study was to expand our understanding of larval-specific ORs, by first characterizing their developmental expression patterns, and then attempting to reduce their expression using RNAi to observe any resulting changes, or absences of changes, in larval behaviour.

Research on the developmental stage at which *Ae. aegypti* larval-specific odorant receptors are most strongly expressed has not yet been published. Existing studies have focused primarily on third and fourth instar larvae when examining OR expression (Bohbot et al. 2007; Matthews et al. 2018; Lutz et al. 2019; Chen et al. 2025). In this study, I characterized the relative expression of OR9 and OR34 across all four larval instars. Although the differences were not statistically significant, OR9 showed its highest relative gene expression in the first and second instars (Figure 3), whereas OR34 expression peaked in the fourth instar larvae (Figure 4). The lack of statistical significance may reflect limited sample sizes, and additional replicates may help determine whether these trends are biologically meaningful. The developmental differences between OR9 and OR34 became even more apparent in later qRT-PCR analyses, as OR34 ultimately was the only gene's transcripts that could be consistently detected. Interestingly, a transcriptomic analysis in *Ae. aegypti,* compared gene expression patterns across six developmental stages, including early and late larvae, early and late pupae, adult females and males (Brent et al. 2013). Although this study did not specifically consider OR expression, it revealed significant shifts in the gene expression patterns between the early and late larval stages (Brent et al.

2013). These findings illustrate the dynamic nature of the larval transcriptome and emphasize the importance of stage-specific profiling of genes, especially during larval development.

A major issue faced throughout this study was the difficulty in detecting *Ae. aegypti* larval-specific OR transcripts. Although protocol optimization improved detection in some cases, many ORs remained undetectable by qRT-PCR. This issue is consistent with previous findings. Mitra et al. (2021) reported that ORs 9, 14, 34, 40, 48, and 58 were expressed at extremely low levels, at less than 1 fragment per kilobase of transcript per million base reads in their RNA-sequenced antenna samples. While these low expression levels help explain the technical challenges encountered here, my study also revealed additional observations about ORs and their relationship to larval behaviour.

Based on previous work, I expected indole to activate OR9 and OR58 (Bohbot et al. 2011; Ruel et al. 2019) and skatole to activate OR9 and OR34 (Ruel et al. 2019). In the chemotaxis assay conducted after 4 days of feeding larvae bacterial pellets containing OR9 dsRNA, there was a significant decrease in positive chemotaxis responses towards skatole compared to the control ($p\text{-value} \leq 0.05$; Figure 9). This finding supports the previous studies' conclusions that OR9 interacts with skatole. However, there was no significant change in the positive chemotaxis response towards indole compared to the control (Figure 9). This finding contrasts with an earlier electrophysiological study; Bohbot et al. (2011) reported that OR9 responds to indole, and Ruel et al. (2019) found that OR9 is sensitive to both skatole and indole. In those studies, *Xenopus* oocytes expressing OR9 were exposed to varying concentrations of the odorants, and responses were measured as changes in current (Bohbot et al. 2011; Ruel et al. 2019). Such assays provide valuable starting points for

behavioural predictions, but because they isolate the receptor from the organism, they do not capture the full complexity of olfactory processing in larvae. Behavioural responses may reflect the combined activity of multiple ORs responding to the same odorant. It is also possible that OR9 was not effectively knocked down in my study. However, because OR9 transcripts were inconsistently detectable by qRT-PCR, I was unable to confirm whether RNAi-mediated knockdown occurred.

Next, I conducted the chemotaxis assay using larvae fed bacterial pellets expressing OR58 dsRNA. Based on the protein docking analysis completed by Dr. Mitchell-Koch, OR58 was predicted to bind indole (Figure 5). AutoDock Vina models generated from Alpha-fold2 structures showed indole positioned closely within the core of the simulated OR58 transmembrane domain, suggesting potential ligand affinity (Del Mármol et al. 2021). However, the chemotaxis assay revealed no behavioural differences between larvae treated with OR58 dsRNA and those treated with the GUS control (Figure 10). This lack of a behavioural difference may indicate that OR58 was not effectively knocked down, or that OR58 does not mediate behavioural responses to skatole or indole. Unfortunately, I was unable to obtain qRT-PCR data to confirm whether RNAi-mediated knockdown occurred.

Finally, chemotaxis assays were performed on mosquito larvae treated with OR34 dsRNA by two different methods. In the first method, larvae ingested bacteria pellets expressing OR34 dsRNA. This treatment produced a significant reduction ($p\text{-value} \leq 0.05$) in the proportion of larvae showing positive chemotaxis to skatole compared to the GUS dsRNA control (Figure 11). Such a reduction suggests that OR34 dsRNA may be successfully lowered through RNAi, resulting in decreased levels of the OR34 protein.

To determine whether OR34 expression decreased after ingestion of OR34 dsRNA, qRT-PCR analyses were performed. No significant decrease in OR34 transcripts was detected in the dsRNA-treated larvae (Figure 6). The lack of statistical significance may be partly due to the small sample sizes used, and more importantly, to the sensitivity limits of the PCR assay. Because the Ct values for OR34 were >35, they fall near the lower detection limit of the instrument, where small errors can be greatly amplified and increase variability in the results. Further optimization could include pooling more larvae to increase cDNA yield, switching to Taq-Man PCR with higher-sensitivity sequence-specific probes, and/or using a more sensitive platform such as digital droplet PCR. Nevertheless, the loss of OR34 function, accompanied by a marked decrease in attraction to skatole, supports the idea that OR34 contributes to skatole detection. These findings imply that skatole is likely one of the ligands that interacts with OR34, and the behavioural effect observed in its absence reinforces this relationship. Although no prior studies have described this interaction, the protein docking analysis conducted by Dr. Mitchell-Koch predicted that skatole can bind to OR34, and the behavioral results from the chemotaxis assay are consistent with this predicted binding (Figure 5).

A novel RNAi delivery method was tested next to evaluate its ability to induce gene knockdown. Previous studies have reported success in RNAi-mediated knockdown in *Ae. aegypti* larvae using soaking-based dsRNA delivery (Singh et al. 2013; Bona et al. 2016; Arshad et al. 2021). In this study, I aimed to determine whether adding the surfactant, Silwet L-77, could enhance dsRNA penetration through the larvae's lipid-coated cuticle, an approach not previously examined in the literature. In the chemotaxis assay conducted on larvae soaked in deionized water containing dsRNA, there was no

difference in the chemotactic responses between larvae soaked in OR34 dsRNA and those treated with the GUS dsRNA control. Likewise, no difference was observed between the "no chemical" control and the skatole treatment (Figure 12). As noted earlier, this may reflect degradation of the skatole stock, previously diluted in DMSO weeks prior. The knockdown of OR34 gene expression was evaluated using qRT-PCR; unexpectedly, there was an increase in the relative OR34 expression in the treated larvae compared to the control, although not statistically significant (Figure 7).

Larvae soaked with 0.0125% Silwet showed a significant increase in positive chemotaxis to the negative control odorant, containing only DMSO (Figure 8; $p \leq 0.05$). As suggested previously, the knockdown of OR34 may have increased the larvae's sensitivity towards DMSO, which may interfere with the larvae's detection of skatole. The addition of Silwet, however, led to a visible difference in the larvae's overall behavior and to mortality in some larvae 1-2 days post-soaking. There was no statistical significance in the relative OR34 gene expression results obtained using qRT-PCR (Figure 8). For the reasons previously mentioned, further optimizations to the analysis of gene expression would be advantageous to determine whether any of the trends in gene expression observed were biologically meaningful and to increase confidence in results.

Overall, this study aimed to advance our knowledge of odorant receptors specific to the larval stage in *Ae. aegypti* mosquitoes. Through the knockdown of OR genes using RNAi and a chemotaxis assay, there is evidence that OR9 and OR34 contribute to olfactory-mediated responses in larvae, more specifically, towards the environmentally relevant odorant skatole. Due to the complexity of odorant receptors and their corresponding ligands, further research is needed to fully characterize the dynamics of

these relationships in *Ae. aegypti* larvae. These findings highlight the underexplored larval olfaction as a promising target for mosquito control strategies. The identification of attractive odorants will be efficacious in the development of larval trapping strategies that aim to attract larvae to an area or device (Barrera 2022). The ability to disrupt olfactory pathways will aid in the development of novel mosquito control strategies aimed at reducing the ability of larvae to escape predation or find food, ultimately reducing their populations. Future work integrating improved OR gene expression detection will be crucial in providing a more complete understanding of odorant receptor-ligand relationships and corresponding olfactory-mediated behaviour to enhance control of the *Aedes aegypti* mosquito.

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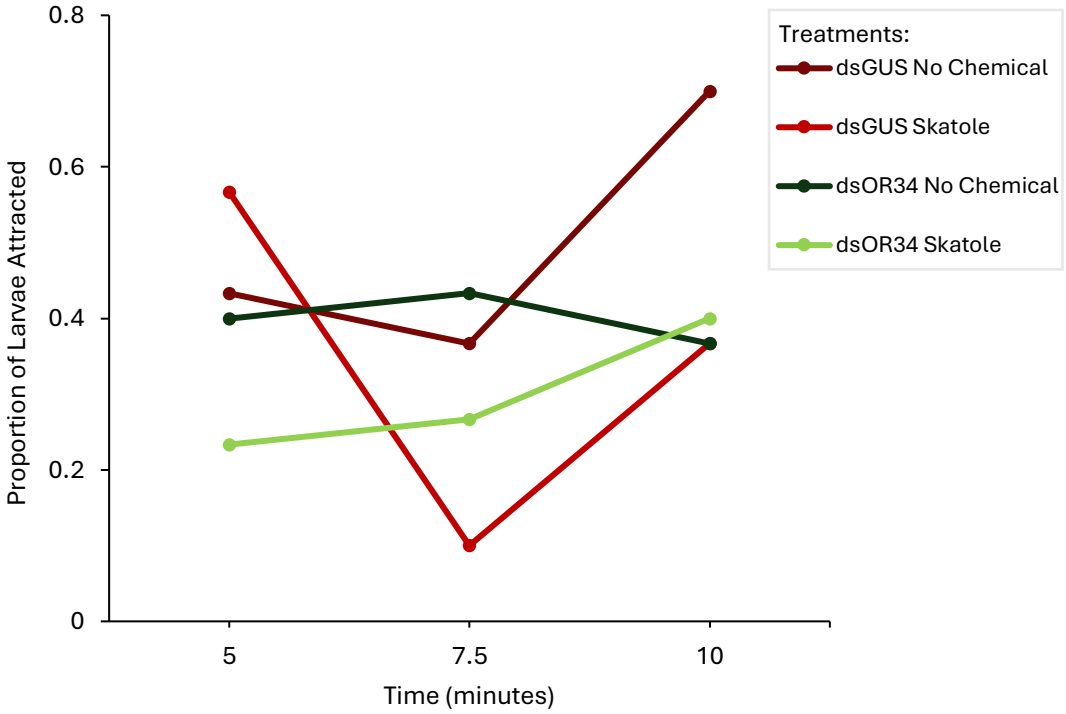
Appendix A



Mosquito Larvae Chemotaxis Over Time: Ingestion of dsGUS vs. dsOR34. Gene knockdown was RNAi-mediated, facilitated through bacterial pellet ingestion containing dsRNA of OR34 and GUS (control) genes for 4 days. The values represent the proportion of 30 *Ae. aegypti* larvae showing positive chemotaxis towards skatole, at the three time points examined.