ABSTRACT

Chemosensory receptor annotation and characterization in *Aedes albopictus*

Garrett McClain Ray

Director: Jason Pitts, Ph.D.

Chemoreception in insects is modulated by receptors expressed in sensory neurons that detect chemical cues in the environment, allowing insects to make sense of the world around them. The chemosensory machinery of the Asian tiger mosquito, *Aedes albopictus*, has been relatively understudied when considering its role as a competent disease vector and incredibly potent invasive species. Some of the greatest gaps in our current knowledge of *Ae. albopictus* chemosensation consist of the protein sequence for chemoreceptors, which of these receptors are expressed in different tissues, and what chemicals activate these receptors to modulate mosquito behavior. The present studies attempt to fill in these gaps. The first study consists of a complete manual reannotation of the *Ae. albopictus* chemosensory genome, including odorant receptors, ionotropic receptors, gustatory receptors and odorant binding proteins. In addition, this study attempts to quantify expression of genes in the tarsal segments and labellum of *Ae. albopictus* through an RNA sequencing experiment. These appendages appear to specialize in contact chemosensation due to the high concentrations of gustatory receptor transcripts present in our RNA sequencing dataset. The second study presented here is the deorphanization of an ionotropic carboxylic acid receptor using an odorant response assay in a heterologous cell system. This receptor, AalbIR75e, is potently activated by nonanoic acid in a concentration-dependent manner. The role of carboxylic acids in mosquito host-seeking behavior is well documented, therefore AalbIR75e may play a role in modulating blood feeding. The reannotation of the *Ae. albopictus* genome and deorphanization of AalbIR75e should serve as a foundation for future functional and behavioral studies of *Ae. albopictus* chemosensation.

APPROVED BY DIRECTOR OF HONORS THESIS:

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Dr. Jason Pitts, Department of Biology

APPROVED BY THE HONORS PROGRAM:

_____

Dr. Al Beck, Director

Date: _____

CHEMOSENSORY RECEPTOR ANNOTATION AND CHARACTERIZATION IN *AEDES ALBOPICTUS*

A Thesis Submitted to the Faculty of

Baylor University

In Partial Fulfillment of the Requirement for the

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By

Garrett McClain Ray

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DEDICATION

Honors thesis by Garrett McClain Ray, dedicated

To,

My Family,

Supportive Friends,

and

Encouraging Mentors

CHAPTER ONE

Introduction: Aedes albopictus, disease transmission, and chemosensation

Mosquito species such as *Aedes aegypti* and *Anopheles gambiae* are well-known vectors for arboviruses and parasites that cause debilitating diseases, affecting human health, society, and economics in endemic countries. Among the viruses transmitted by mosquitoes are yellow fever, dengue fever, chikungunya, and zika. Dengue fever alone is estimated to infect 390 million people per year, with approximately 4 billion people at risk for infection at any time (Bhatt et al., 2013; Brady et al., 2012). Unlike the malaria parasite, which tends to stop harming affected individuals after the infection is over, arboviruses can have lingering effects. Multiple arboviral diseases impart chronic somatic conditions and neurological deficits to patients who survive the initial infection (Carson et al., 2006; Gupta et al., 2009; Sejvar et al., 2006; Sejvar, 2007; Siam & Meegan, 1980). One study estimated that yellow fever, Japanese encephalitis, chikungunya, and Rift Valley fever infections in 2005 will have cost between 300 thousand and 5 million disability-adjusted life years (LaBeaud et al., 2011). Among the most competent vectors for mosquito-borne viruses are a handful of mosquitoes from the *Aedes* genus. *Ae. aegypti* is undoubtedly the most infamous and most extensively studied vector in this genus due to its association with the yellow fever virus. However, *Ae. aegypti* is not the only *Aedes* species that is capable of transmitting arboviruses. *Ae. albopictus,* the Asian tiger mosquito, has been implicated in the spread of dengue in Asia and is a competent

vector for 26 viruses (Paupy et al., 2009; Powell, 2018). Despite the ability of *Ae. albopictus* to transmit multiple pathogens, it has remained relatively understudied when compared to *Ae. aegypti.* However, it has been demonstrated that *Ae. albopictus* is displacing *Ae. aegypti* in many places around the world (Bagny, Delatte, Elissa, et al., 2009; Bagny, Delatte, Quilici, et al., 2009; Kamgang et al., 2013; Kaplan et al., 2010; Leon Philip Lounibos & Kramer, 2016; L.P. Lounibos et al., 2001). If this trend continues, *Ae. albopictus* may become a more threatening disease vector than *Ae. aegypti*, making research on *Ae. albopictus* increasingly more important. One critical line of research examines how mosquitoes respond to sensory stimuli. Like all insects, mosquitoes detect chemical cues in the environment using chemoreceptors that are expressed in sensory neurons and modulate behaviors such as oviposition and host-seeking, which are vital to the survival of the species and also impact their ability to transmit disease (Hughes et al., 2010; Matthews et al., 2016). As with other types of research, chemosensory research into *Ae. albopictus* has trailed that of *Ae. aegypti.* Some of the greatest gaps in our current knowledge of *Ae. albopictus* chemosensation consist of the protein sequence for chemoreceptors, which receptors are expressed in different tissues, and what chemicals activate these receptors to modulate mosquito behavior. The present studies attempt to fill in these gaps. The first study consists of a complete manual reannotation of the *Ae. albopictus* chemosensory genome, including odorant receptors, ionotropic receptors, gustatory receptors and odorant binding proteins. In addition, this study attempts to quantify expression of genes in the tarsal segments and labellum of *Ae. albopictus* through an RNA sequencing experiment. The second study presented here is the deorphanization of an ionotropic carboxylic acid receptor using an odorant response

assay in a heterologous cell system. This receptor, AalbIR75e, is potently activated by nonanoic acid in a concentration-dependent manner. The role of carboxylic acids in mosquito host-seeking behavior is well documented, therefore AalbIR75e may play a role in modulating blood feeding. The reannotation of the *Ae. albopictus* genome and deorphanization of AalbIR75e should serve as a foundation for future functional and behavioral studies of *Ae. albopictus* chemosensation.

As noted above, chemosensation allows mosquitoes to detect chemical cues in the environment, which regulates mosquito behavior. The chemosensory machinery in mosquitoes, and all flies, are associated with odorant receptor neurons (ORNs) that are housed within sensilla (Vosshall & Stocker, 2007). These ORNs extend their dendrites towards the free surface of the sensillum and are covered with chemosensory receptors that recognize semiochemicals in the environment. The binding of chemicals to these receptors has been shown to mediate many important biological behaviors such as oviposition site selection and host-seeking (Hughes et al., 2010; Matthews et al., 2016). The chemosensory mechanism that modulates host-seeking behaviors is particularly important due to the role of this behavior in the transmission of mosquito-borne diseases (Day, 2005). Studies have shown that host-seeking female mosquitoes utilize multiple chemosensory mechanisms (DeGennaro et al., 2013; Gillies, 1980; McMeniman et al., 2014). Further evidence supporting the role of chemosensation in host-seeking behavior is provided by studies detailing the down-regulation of certain chemoreceptors during periods of host-seeking repression that occur after a blood meal has been taken (Klowden, 1981; Rinker et al., 2013; Siju et al., 2010; Takken et al., 2001; Taparia et al., 2017).

The behavior-mediating receptors present on ORNs are classified into three major gene families: odorant receptors (ORs), gustatory receptors (GRs), and ionotropic receptors (IRs). ORs are a family of seven-transmembrane receptors that were identified in *Drosophila melanogaster* in 1999 (Gao & Chess, 1999; Vosshall et al., 1999). OR-mediated chemosensation requires the creation of a heteromeric complex between some number of OR*X*s and the OR co-receptor, Orco. Since their discovery, ORs have been implicated in many different behaviors and are highly divergent, possibly due to their ancient origin (Robertson et al., 2003). ORs have been shown to aid in the selection of oviposition sites through the detection of indolic compounds (Hughes et al., 2010). ORs have also been shown to detect volatile alcohols, ketones, and phenols (Bohbot & Dickens, 2009; Carey et al., 2010; McBride et al., 2014). ORs are also implicated in host-seeking behaviors. OR4 in *Aedes aegypti* was shown to be selective for sulcatone, a compound present in human sweat which may provide mosquitoes with a preference for human odors (McBride et al., 2014). Additionally, mosquitoes with a defective Orco protein lose their ability to distinguish human odors from that of other hosts (DeGennaro et al., 2013).

GRs also play important roles in chemosensation. Unlike ORs and IRs, which detect volatile compounds, GRs have typically been found to detect soluble compounds and carbon dioxide (Jones et al., 2007; Kwon et al., 2007). Soluble compounds, such as sugars, can be detected by GRs, which may modulate the sugar-feeding behaviors observed in male mosquitoes (Kent et al., 2008). Carbon dioxide sensation by GRs has been studies extensively. GR sensation of carbon dioxide mediates the upwind flight behavior that helps mosquitoes find blood meals (Healy & Copland, 1995). Carbon

dioxide, human odor, and heat seem to work in conjunction to modulate blood feeding behavior, with each component being necessary (McMeniman et al., 2014). When carbon dioxide detection was disrupted by a loss of function mutation in GR3 there was a marked reduction in host-seeking behavior, further demonstrating the role of carbon dioxide in blood-feeding (Raji et al., 2019). GRs also seem to play a role in DEET's contact repulsion of mosquitoes (Sanford et al., 2013).

Finally, the IRs are set of highly conserved olfactory receptors that evolved from ionotropic glutamate receptors (Rytz et al., 2013). These receptors seem to be narrowly tuned to the amine and carboxylic acids functional groups, both of which are extremely common in nature (Pitts et al., 2017). However, IRs are not limited to chemical detection and have demonstrated effects on many different behaviors throughout the insect life cycle, such as thermal and hygrosensation during the larval stage (Enjin et al., 2016; Ni et al., 2016). IR chemosensation in mosquitoes utilizes three different co-receptors: IR8a for carboxylic acid sensation, while IRs 25a and 76b mediate polyamine sensitivity (Hussain et al., 2016; Pitts et al., 2017). IRs are also associated with host-seeking behavior. Lactic acid, a component of human sweat, is one of the most potent attractants of mosquitoes and its sensation seems to be modulated by IRs, as IR8a knock-outs demonstrated no attraction to lactic acid (Davis, 1984; Dekker et al., 2002; Raji et al., 2019; Steib et al., 2001). Other carboxylic acids that are components of human sweat have also been implicated in host-seeking behavior (Dekker et al., 2002). Pitts et al. have shown that IRs 75k and 75l in *Anopheles gambiae* are receptive to octanoic and nonanoic acids (Pitts et al., 2017). Given the effect of lactic acid, and carboxylic acids in general, on host-seeking behavior, elucidation of IRs remains an important area of study.

Our studies have focused on the role of IRs in *Ae. albopictus. Ae. albopictus* is considered to be one of the most successful invasive species, spreading from their native range in south-east Asia to areas as distant as Africa and the Americas (Lombardo et al., 2017; Paupy et al., 2009). This puts many people at risk for disease transmission from *albopictus* and makes them an important species to research. We began by building upon the work of Lombardo et al. who collected RNA sequencing data on the primary olfactory organs, antenna and palps, in *Ae. albopictus* (Lombardo et al., 2017). We built upon this study by collecting RNA sequencing data for the labella and tarsi, which are considered secondary olfactory organs in adult mosquitoes. While analyzing this data, we recognized that the *Ae. albopictus* genome assembly was of lower quality compared to the extremely well-annotated *Ae. aegypti* genome (Matthews et al., 2016). Thus we have attempted to reannotated the entire chemosensory receptor repertoire of *Ae. albopictus*, using the *Ae. aegypti* gene families as a reference point. This reannotation will allow future studies to proceed more efficiently and will facilitate the identification of additional putative receptors in *Ae. albopictus.* While the reannotation of the chemosensory "receptome" is undoubtedly important work, studies on receptor function are equally important. Characterizing the cognate ligands of IRs allows for behavioral studies to determine the role of these receptors as mediators of important life history traits. In the present study, we have limited our investigation to the most highly conserved IRs, as these are likely to be involved in common behaviors across *Aedes* species like host-seeking. Following identification, we utilize a technique called two electrode voltage clamping (TEVC) to perform odorant response assays in a heterologous cell system. Using this assay, we can determine the response profiles of chemoreceptor

proteins to a wide array of chemical compounds by measuring changes in the flow of ions (i.e. current) across the cell membrane. This is a well-established methodology that is a relatively simple, yet efficient way to determine both the efficacy and potency of chemical ligands for mosquito chemoreceptors (Bohbot & Dickens, 2009; Huff & Pitts, 2019; McBride et al., 2014; Pitts et al., 2017).

CHAPTER TWO

RNA Sequencing and Chemosensory Gene Annotation

Introduction

Like all flies, *Ae. albopictus* possesses three major chemoreceptor families, housed in odorant receptor neurons, that detect relevant chemical stimuli in the environment and modulate mosquito behavior (Suh et al., 2014; Vosshall & Stocker, 2007). The role of many receptors is still being characterized using *Xenopus laevis* heterologous cell systems (Bohbot & Pitts, 2015). The first of these gene families are the odorant receptors (ORs). ORs are generally dimeric, although some trimers have been reported, in structure, with the OR co-receptor (Orco) combining with another OR to form a functional complex (Goldman et al., 2005; Suh et al., 2014). ORs are among the quickly evolving genes in the mosquito genome, resulting in a wide variety of chemical ligands (Bohbot & Dickens, 2009; Carey et al., 2010; Hughes et al., 2010; McBride et al., 2014). Due to their divergent nature, ORs have been implicated in many different behaviors. *AaegOR4,* a receptor for the volatile compound present in human odor, has been implicated in the high degree of anthropophily displayed by *Ae. aegypti* (McBride et al., 2014). Another study found that *OR10* in *Culex quinquefasciatus* was sensitive to skatole (Hughes et al., 2010). The researchers believe that skatole, and other indolic compounds are important oviposition cues. Finally, *Anopheles gambiae OR29* is sensitive

to the plant-emitted volatile linalool (Huff & Pitts, 2019). These *ORs* may play a role in the detection of nectar sources for nonblood-feeding mosquitoes. While extensive knock-out work is necessary to confirm the predicted phenotypes of these receptors, the receptor deorphanization experiments described point to the wide range of behaviors *ORs* modulate.

The second gene family consists of the variant ionotropic glutamate receptors (IRs). IRs are oldest chemosensory gene family and are highly conserved when compared to the other chemosensory gene families (Croset et al., 2010; Rytz et al., 2013). Along with ORs, IRs specialize in the reception of volatile chemicals in the environment. IRs also form heteromeric proteins. Three different co-receptors have been identified within IR family. IR8a is widely associated with carboxylic acid reception, while IRs 25a and 76b mediate polyamine sensitivity (Ai et al., 2010, 2013; Hussain et al., 2016; Pitts et al., 2017; Raji et al., 2019). While carboxylic acids and polyamines are the most salient volatile chemicals that IRs are tuned for, IRs have also been implicated in hygrosensation, amino acid detection, and thermosensation (Enjin et al., 2016; Ganguly et al., 2017; Knecht et al., 2017; Ni et al., 2016). Among the most important roles of IRs in mosquitoes is the detection of lactic acid (Raji et al., 2019). Lactic acid is a metabolic byproduct of anaerobic respiration present in sweat and is the most potent known attractant of mosquitoes (Acree et al., 1968; Davis, 1984; Dekker et al., 2002; Steib et al., 2001). While the receptor for lactic acid has not been elucidated, IR8a knockouts lose sensitivity to lactic acid, suggesting that IRs are responsible for lactic acid reception (Raji et al., 2019)

Gustatory receptors (GRs) are the final gene family that contributes to the chemosensory machinery of insects. Unlike ORs and IRs, GRs tend to detect solubilized chemicals and carbon dioxide (Jones et al., 2007; Kwon et al., 2007). Among the solubilized compounds detected by GRs are sugars, which may help male mosquitoes identify suitable nectar sources to feed on (Kent et al., 2008). The effect of carbon dioxide reception by GRs is well documented. Carbon dioxide sensation mediates upwind flight behavior, leading mosquitoes to possible blood meals (Healy & Copland, 1995). Additionally, carbon dioxide sensation seems to be required for host seeking, as GR3 mutants displayed markedly reduced host-seeking behavior (McMeniman et al., 2014; Raji et al., 2019).

While the deorphanization of chemoreceptors is important, other information about these receptors is also valuable to researchers. Expression data that identifies which receptors are present in tissues can give researchers clues to the phenotypes associated with the receptor. In *Ae. aegypti*, this expression data is readily available because of an extensive *RNA* sequencing experiment performed by the Vosshall lab (Matthews et al., 2016). In addition to this *RNA* sequencing data, this paper also reannotated the already impressive *Ae. aegypti* genome. In contrast, there is only one RNA sequencing experiment in *Ae. albopictus,* which examined the expression level of receptors in the antennae and maxillary palps (Lombardo et al., 2017). In addition to localizing receptor expression, expression data can be used to directly monitor the changes in receptor expression over time. One such study examined the expression of receptors using RT-PCR to determine the expression of receptors following blood feeding (Taparia et al., 2017). Mosquitoes demonstrate a distinct inhibition of blood-feeding behavior after

successfully taking a blood meal (Siju et al., 2010; Takken et al., 2001). The study by Taparia et al, identified 6 IRs that were down-regulated after mosquitoes took a blood meal, suggesting that they play a role in host-seeking behaviors.

The incredibly polished chemosensory genome available for *Ae. aegypti* is a valuable resource for researchers. In comparison, the *Ae. albopictus* genome is not as well maintained and has not been manually annotated. Additionally, only a single *RNA* sequencing experiment has ever been completed in *Ae. albopictus.* In this paper, we detail our manual annotation of the three major chemosensory gene families and the OBPs in *Ae. albopictus*. We also employed *RNA* sequencing to quantify the expression of chemoreceptors in the secondary olfactory organs, tarsi and labellum, of *Ae. albopictus.*

Methods

Mosquito care and dissections

For these experiments, *Ae. albopictus* adults were obtained from the Pitts lab colony. Mosquitoes were reared in the Baylor University Tropical Disease Biology Lab at 27°C , 70% relative humidity, and a 12:12 light:dark cycle. Larvae were fed a diet of ground koi fish food and yeast. Adults were provided on a 10% sucrose solution *ad libitum* until they were collected for dissection two to six days post-emergence (Lombardo et al., 2017). Upon collection, adults were cold anesthetized by placing in a -20°C freezer. Tarsi (distal five segments of legs) and labellar lobes (distal tips of proboscises), were removed from anesthetized mosquitoes and placed in Trizol reagent to prevent RNA degradation. The dissected tissues were frozen in Trizol® reagent (Life technologies) until the RNA isolation procedure could be completed. Two distinct

samples were derived for each tissue using approximately 500 individual males and 500 individual females, to produce biological replicates for each tissue and sex.

RNA isolation and sequencing

Total RNA was isolated using the Trizol® reagent RNA isolation protocol provided by the manufacturer with a single alteration. Tissues were mechanically homogenized in Trizol® using a pestle prior to the RNA extraction procedure. One change was made to the manufacturer's protocol, which called for the use of chloroform during the RNA extraction procedure. Chloroform was replaced with the relatively stable 1-bromo-3-chloropropane due to the potential danger the autooxidation reaction of chloroform when exposed to air, which produces phosgene and hydrochloric acid, poses for researchers (Clover, 1923; Thowfeequ & Michos, 2012; Vaish et al., 2013). Following RNA extraction, the RNA was precipitated using an isopropanol and glycogen solution. The precipitated RNA was then purified via ethanol precipitation. After decanting, RNA was resuspended in RNAse free water and passed through an RNAse-free polyacrylamide size exclusion column to remove small contaminant and organics (MicroBio Spin 30; BioRad Labs). RNA samples were tested for quality and quantity using a Nanodrop One spectrophotometer (Thermo Fisher Scientific). The isolated total RNA was then sent to a commercial supplier (Novogene, Beijing, China) for library preparation and sequencing. The resulting 150 base-pair, paired end reads were used for expression analysis.

Expression quantification

Prior to quantification, low-quality reads as well as contaminating microbial-derived reads were removed. To quantify the expression of chemoreceptors in tissues, filtered reads were mapped to the existing Foshan *Ae. albopictus* transcriptome available on VectorBase. The paired-end reads were mapped using the Sailfish alignment-free quantification software, with k-mers of 21 bp, an effective read length of 150bp, and sequence-specific bias correction (Patro et al., 2014). The resulting dataset contained read counts from each transcript and expression level, in TPM (transcripts per kilobase per million reads). TPM was used to measure expression level of receptors because it corrects for the length of the transcript, which could artificially inflate or deflate the number of reads that map. Expression data was compared between the two samples to confirm that our results were replicable. This comparison was conducted by using a fold-change logarithm and comparing the resulting expression data (figures 1-9).

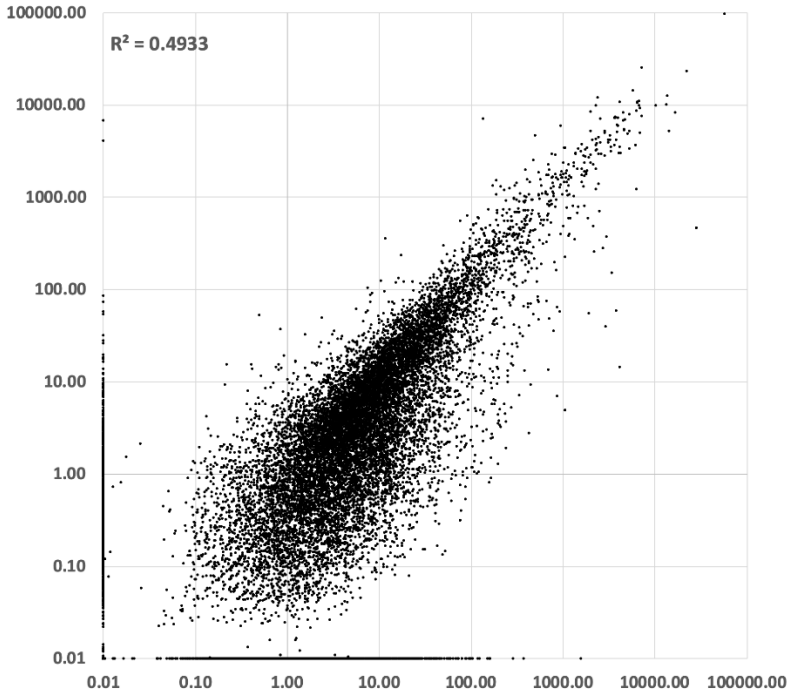


Figure 1: Correlation analysis of all transcripts in our two samples using a fold-change logarithm to quantify expression levels

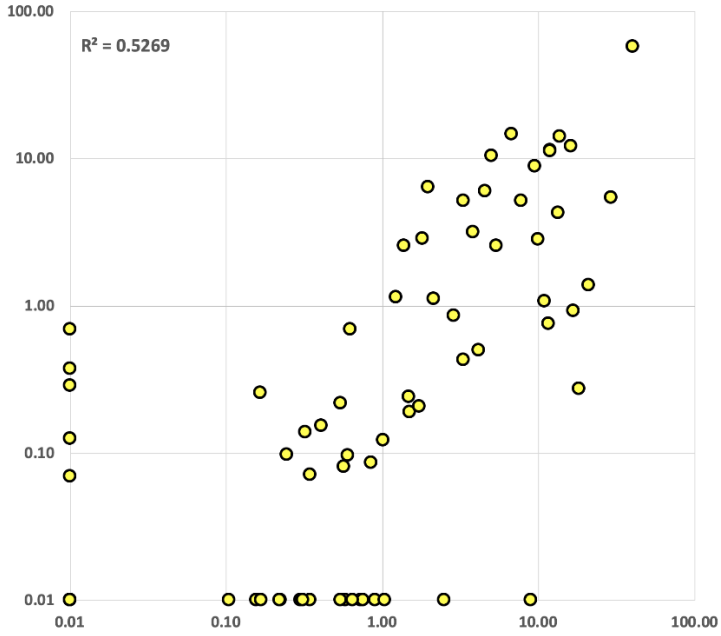


Figure 2: Correlation analysis of OR transcripts in labellar lobes between samples

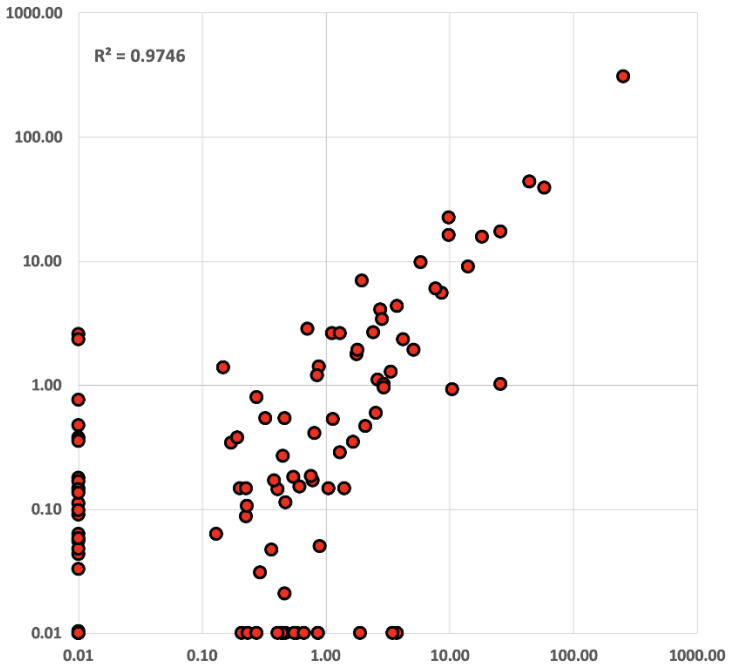


Figure 3: Correlation analysis of IR transcripts in labellar lobes between samples

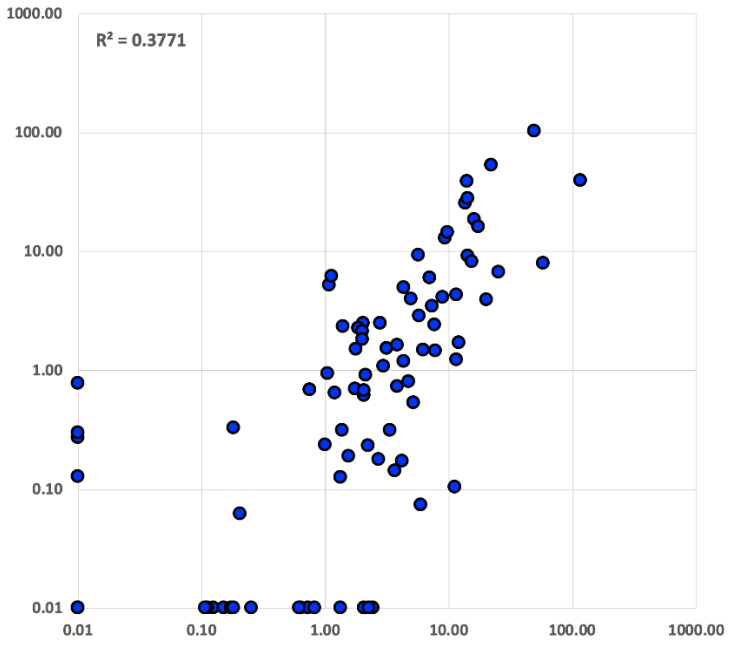


Figure 4: Correlation analysis of GR transcripts in labella lobes between samples

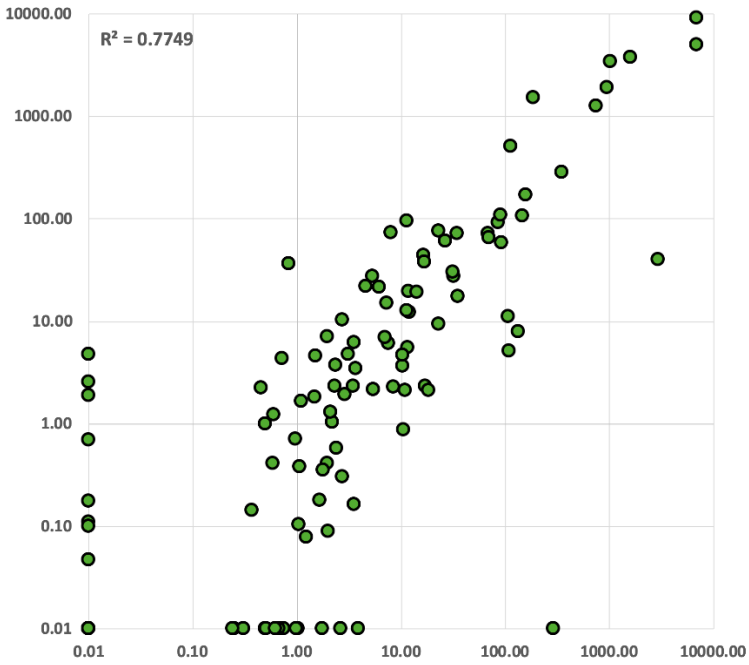


Figure 5: Correlation analysis of OBP transcripts in labellar lobes between samples

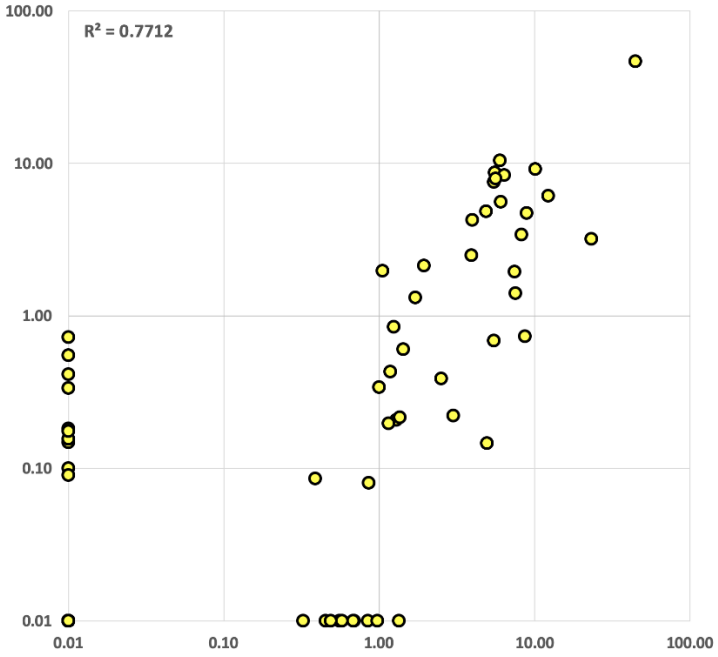


Figure 6: Correlation analysis of OR transcripts in tarsi between samples

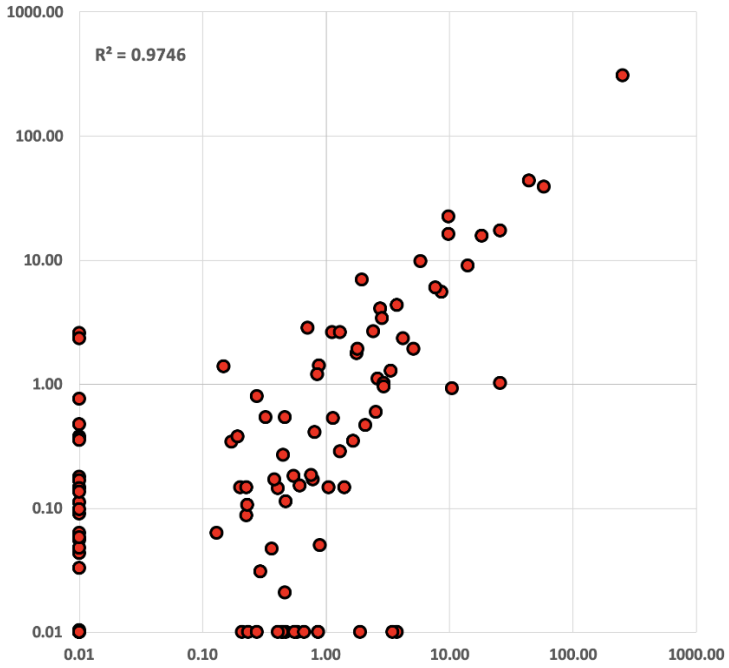


Figure 7: Correlation analysis of IR transcripts in tarsi between samples

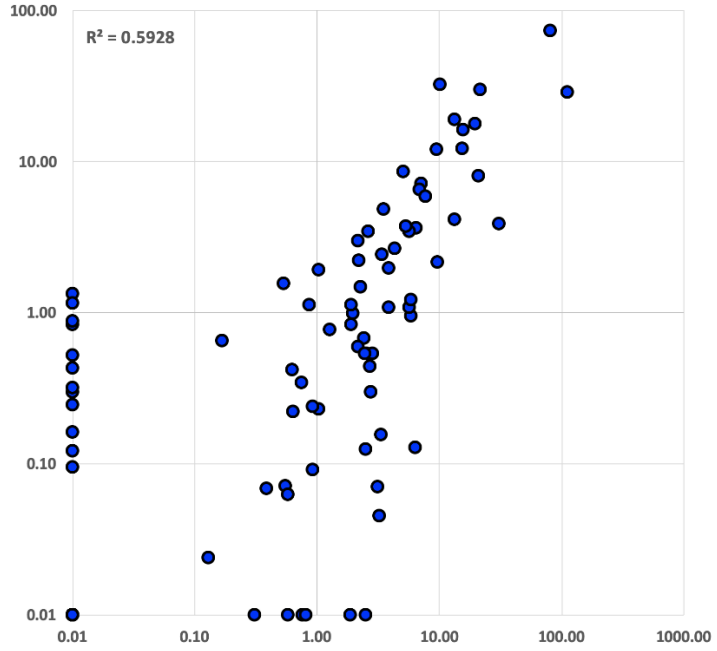


Figure 8: Correlation analysis of GR transcripts in tarsi between samples

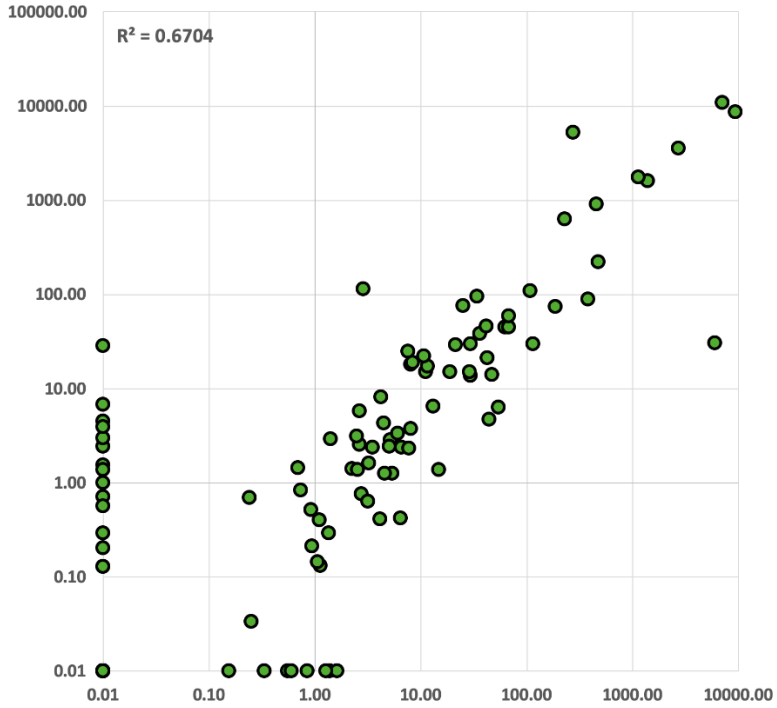


Figure 9: Correlation analysis of OBP transcripts in tarsi between samples

Gene annotation

Ae. albopictus chemosensory gene families were hand-annotated using the *Ae. aegypti* conceptually translated peptide sequences for ORs, IRs, GRs, and OBPs as references. Specifically, *Ae. aegypti* peptide sequences were utilized in tBLASTn queries of the *Ae. albopictus* Foshan assembled genome scaffolds (VectorBase.org). BLAST results included regions of the *Ae. albopictus* genome that contained the highest homologies to the input *Ae. aegypti* peptides. These sequences were used to manually identify and delineate exons encoding *Ae. albopictus* chemoreceptor peptides. The annotated *Ae. albopictus* coding regions were subsequently aligned with the *Ae. aegypti* peptide sequence using BLASTx (NCBI.gov) as a basis for validating new annotations.

Results

Gene annotation

While analyzing the RNA sequencing dataset it became apparent that the chemoreceptors contained within the *Ae. albopictus* transcriptome dataset were incomplete, potentially owing to a reduced quality genome assembly as compared with *Ae. aegypti* (Matthews et al., 2016). One of the major goals of this project was to help improve the condition of the *Ae. albopictus* nucleic acid dataset through the reannotation of the chemosensory genes. To this end, we have completed annotations of 120 ORs, 91 IRs, 84 GRs, and 59 OBPs. Despite the chemosensory gene families being among the most rapidly evolving genes in mosquitoes, we saw remarkable similarity between the *Ae. aegypti* and *Ae. albopictus* peptides (Benton, 2015; Hansson & Stensmyr, 2011; Ramdya & Benton, 2010).

Table 1: The average homology of a gene in each gene family when the *Ae. albopictus* and *Ae. aegypti* proteins were compared using NCBI. Homology was measured using the number of identical amino acids (identities) and the number of structurally/chemically similar amino acids (positives)

	Identities	Positives
ORs	69.5%	82.8%
IRs	75.5%	86.6%
GRs	75.4%	85.4%
OBPs	85.4%	89.7%

RNA sequencing and analysis

RNA sequencing yielded 2 data sets each for male tarsi, male labella, female tarsi, and female labella. The number of reads in each dataset varied from 9,218,741 to 30,764,907. Despite the wide range in read quantities, the quality of each filtered dataset was high (Q30 >91.34), indicating that our samples accurately represent RNA levels in

these tissues. We have used color-coded heat maps to display the average levels of chemoreceptor transcripts in tarsi and labella of both sexes (Figure 10). Both sexes have very similar patterns of transcript abundances across all gene families (Figure 10). Compared to the expression levels in the primary chemosensory organs, antenna and maxillary palps, our tarsi and labellum samples display a higher abundance of GR transcripts, both in terms of the number of GRs expressed and in their relative levels, while also displaying relatively lower levels of ORs and IRs (Lombardo et al., 2017). Our expression data further suggests that some ORs and IRs are specifically localized in the tarsi and labellum, which may reflect their tissue-specific function in sensory neurons (Figure 10). For example, *AalbOR61-N3* and *AalbIR75o* display far greater expression in the tarsi and labella than the antenna or palps. The *AalbIR7* series of homologs also seems to be highly expressed in the labella. Interestingly, we also found a series of opsins that are expressed in the tarsi and labella. Of particular note is a long-wavelength opsin and rhodopsin with expression greater than 6000 TPM and 1000 TPM, respectively, in male and female labella (table 2).

Table 2: Expression level, in TPMs, of select transcripts

Receptor name	Female labellum	Female tarsi	Female antennae	Female palps
AalbOR61-N3	36.25	26.16	.45	0.00
AalbIR75o	104.52	142.83	17.34	8.69
AalbIR7j	22.48	3.60	0.04	0.20
AalbIR7l	5.01	0.65	0.03	0.00
AalbIR7q.2	9.97	0.00	0.30	0.07
AalbIR7q.2-N1	5.30	0.00	0.00	0.00
AalbIR7r.1	3.19	0.00	0.00	0.00
AalbIR7s.1	22.82	0.68	0.30	0.33
AalbIR7z.1	27.90	26.55	0.77	0.00
AalbIR7k.1	2.51	2.80	0.03	0.00
Rhodopsin	1173.73	212.82	437.89	1107.85
Long-wavelength opsin	6158.82	330.66	1297.90	2561.17

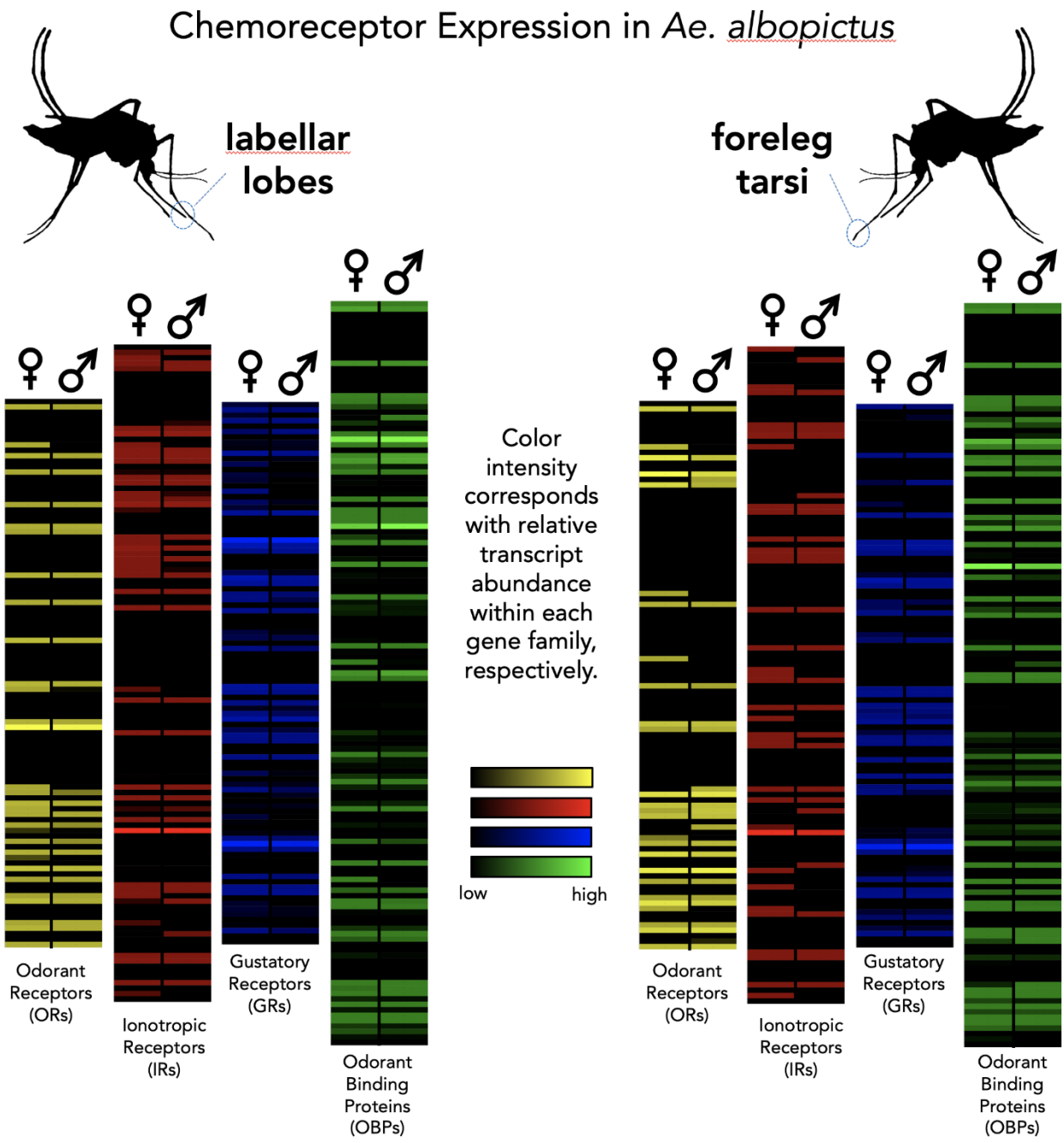


Figure 10: Heat map displaying expression levels of chemosensory genes

Discussion

The main goal of this project was to provide a manually annotated chemosensory genome that can be used for future research. As noted above, we have annotated 120 ORs, 91 IRs, 84 GRs, and 59 OBPs. The receptors that we have annotated displayed a very high degree of conservation when compared with the *Ae. aegypti* peptide sequences. Since evolution tends to either maintain or converge towards things that improve fitness, the most highly conserved receptors are good targets for deorphanization and likely modulate important behaviors in the mosquito. Since these receptors are among the most quickly evolving genes in mosquitoes, it suggests that *Ae. aegypti* and *Ae. albopictus* are more closely related than previously thought (Benton, 2015; Hansson & Stensmyr, 2011; Ramdya & Benton, 2010). If these species are as closely related as our data indicates, it shows that *Ae. albopictus* is likely just as capable of spreading arboviruses as *Ae. aegypti*, even if it hasn't been the major vector for an epidemic yet. Additionally, *Ae. albopictus* has been slowly displacing *Ae. aegypti* by out-competing them across a wide variety of locations, ranging from Florida to islands in the Indian Ocean (Bagny, Delatte, Elissa, et al., 2009; Bagny, Delatte, Quilici, et al., 2009; Kamgang et al., 2013; Kaplan et al., 2010; Leon Philip Lounibos & Kramer, 2016; L.P. Lounibos et al., 2001). As *Ae. aegypti* continues to be displaced, it is possible that *Ae. albopictus* could become a more potent vector of disease by having a larger range of habitation. This possibility alone shows that *Ae. albopictus* research is of higher priority than it is currently given in the scientific community.

One of the most interesting findings of our RNA sequencing study was the significantly higher expression of GRs in the secondary chemosensory appendages, tarsi

and labella. The high expression of GRs in the tarsi and labella is likely due to their role in sensing solubilized chemicals. Tarsi and labella are among the only parts of a mosquito that contact a surface while landed, meaning that they must necessarily have higher expression of GRs for mosquitoes to properly detect solubilized chemicals. The evident role of tarsi and labella in solubilized chemical sensation, and relative lack of GR expression in antenna and maxillary palps, suggests that the tarsi and labella should be referred to as the primary contact chemosensory organs, rather than secondary chemosensory organs.

Finally, the expression of opsins in the labella and tarsi of *Ae. albopictus* was also surprising. Opsins are most well-known for their role as photoreceptors in the visual system, however they are clearly not fulfilling that role in these tissues. Which begs the question: why are they present in these tissues? While it is too early to speculate on the role of opsins within these tissues, opsins have been associated with multiple light-independent sensory modalities including temperature sensation, hearing, and proprioception (Leung & Montell, 2017; Zanini et al., 2018). Given the major roles these appendages play in mechanosensation and contact chemosensation, opsins expressed in these tissues could be acting as pressure sensors, indicating position or touch, or even as heteromeric subunits of gustatory receptors. It is common knowledge that applying gentle pressure on a closed human eye produces a phosphene, an impression of light without light passing to the retina. This shows that opsins in our eyes can respond to mechanical stimulation in addition to photo-stimulation. It is possible that the opsins present in the labella and tarsi of mosquitoes can detect pressure in the same way. Moreover, their potential roles in taste sensation, in conjunction with GRs, cannot be ruled out. These

hypotheses will be interesting to explore in future studies. Technical approaches could include cell-specific localization of opsins within these tissues using *in-situ* hybridization, single sensillum recordings of sensory neurons, and CRISPR-based gene editing. Experiments of this nature could provide novel mechanisms that help explain touch and taste perception in mosquitoes.

CHAPTER 3

IR75e deorphanization

Introduction

Mosquitoes are known vectors of arboviruses such as yellow fever virus, dengue fever, chikungunya, and Zika. Dengue alone is estimated to infect 390 million people per year, and 3.97 billion people are estimated to be at risk for dengue infection (Bhatt et al., 2013; Brady et al., 2012). To reduce the disease burden that mosquitoes confer to regions with endemic arboviruses, it is necessary to understand the mechanisms by which mosquitoes spread these diseases. It has been demonstrated that mosquito-mediated transmission of arboviruses primarily occurs through the female-specific behavior of blood feeding (Day, 2005; Huang et al., 2019; Schneider & Higgs, 2008). To find blood meals effectively, mosquitoes possess a variety of sensory receptors that tuned to detect chemical cues in the environment, also known as chemoreceptors. These chemoreceptors are expressed in sensory tissues such as the antenna, maxillary palps, labellum, and tarsi (Lombardo et al., 2017; Matthews et al., 2016). There are three families of chemoreceptors in insects: the odorant receptors (ORs), gustatory receptors (GRs), and ionotropic receptors (IRs) (Benton et al., 2009; Carey et al., 2010; Kent et al., 2008). Characterizing the function of chemoreceptors, through determination of cognate ligand-receptor pairs, allows for future behavioral studies to determine the effect of a chemical

on mosquito behavior. Despite the efforts of many researchers, many ligand receptor-pairs in mosquitoes have yet to be elucidated.

With regards to the modulation of host-seeking behaviors, the IR family of chemoreceptors seem to hold the most promise. Lactic acid, a volatile carboxylic acid compound found in human sweat, has proven to be a powerful attractant of mosquitoes and seems to convey some species a preference for human blood meals (Acree et al., 1968; Dekker et al., 2002; Eiras & Jepson, 1991; McMeniman et al., 2014; Siju et al., 2010; Steib et al., 2001). Due to its role as a mosquito attractant, lactic acid has been studied extensively. Lactic acid sensation has been shown to require IR8a, as IR8a knock-outs displayed little attraction when lactic acid was present (Raji et al., 2019). Given the role of lactic acid as a powerful attractant of mosquitoes, other carboxylic acids may also be implicated in host-seeking behaviors (Dekker et al., 2002). Many other carboxylic acids are found in human sweat and may contribute to the mosquitoes' demonstrated attraction to sweat (Bernier et al., 2000; Cork & Park, 1996; Liu et al., 2013). Due to the possible role of carboxylic acid receptors on mosquito host seeking behaviors, these receptors are valuable targets for ligand-receptor pair deorphanization.

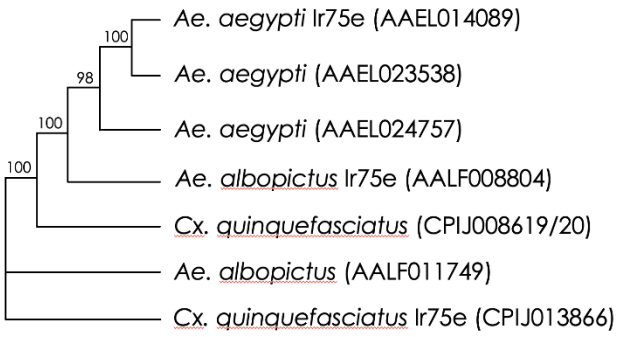
Despite the known role of carboxylic acids on mosquito attraction, there has been relatively little published work regarding the characterization of these receptors. IR8a seems to act as a necessary co-receptor for IR-mediated sensation of carboxylic acids (Pitts et al., 2017; Raji et al., 2019). Pitts et al. have shown that IRs 75k and 75l in *Anopheles gambiae* are tuned to two carboxylic acids, octanoic and nonanoic acids (Pitts et al., 2017). The highly conserved nature of IRs between mosquito species and genera indicate that homologs of these receptors may play a similar role in other mosquitoes. In

addition, the striking similarity between receptors in the IR75 series of receptors could indicate a shared sensitivity toward carboxylic acids. The present study investigates the selectivity, affinity, and potency of putative carboxylic acid receptor IR75e in *Aedes albopictus (*AalbIR75e). AalbIR75e selectivity was determined using an odorant response assay in a Xenopus heterologous cell system. AalbIR75e was maximally activated by nonanoic acid and activated to a far lesser degree by octanoic acid. Nonanoic acid activates AalbIR75e in a concentration-dependent manner. In addition to the high magnitude of activation caused by nonanoic acid, AalbIR75e displayed a high affinity for the compound, suggesting that we have found a cognate ligand-receptor pair.

Methods

Phylogenetic analysis

Homologs of AalbIR75e were identified in the genomes available on NCBI using the tBLASTn feature. Multi-sequence alignment was achieved using the Geneious Prime software (Biomatters Limited, USA). Phylogenetic tree construction was performed using the Neighbor-Joining method with 1000 bootstrap pseudoreplicates (figure 11-12).



	AAEL014089	AAEL023538	AAEL024757	AALF008804	CPIJ008619/20	AALF011749
AAEL023538	87					
AAEL024757	81	79				
AALF008804	82	77	76			
CPIJ008619/20	71	68	69	72		
AALF011749	43	43	43	44	46	
CPIJ013866	38	37	37	38	39	59

Figure 11: Phylogenetic analysis of AalbIR75e (top). Alignment of AalbIR75 with other chemoreceptors (bottom).

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AaegIr75e   MRSPAAIIVFLAFSMSVEASTSDLITVATDLIQCLKLPVQISVISCWSESDKLRFWKEINSF-TAVRFSSENQVNLPWSDRDQHQSLLVIDADCSKSKDL   99
AalbIr75e   MGSLNVILVLLVLSKTVLGSSPDHISAVSDLIHCLKLPVQVSVLSCWSESEKFQFLKEINAI-AAVRFSIEDQQNLPWNDRNLHQNLLVIDVGCPRSKEL   99
CquiIr75e   ----------------------------------------MQDGCWTDHEKTFFWRELASIPAAVRFSGLDVGDLPWDDPNQHRNLLVVDASCPGTKRL   59

AaegIr75e   LRSAGQLLYNRVKWIIINSKNLNCRKFLKFFDDLKLLISNEVYYICEADGVQGFAIKQVYRLSLATEVIEETFGYWNKGMIVNLQHIK-HSSVRRQNLKH  198
AalbIr75e   LRSAGQLLYYRVKWIAVNINDPDCVKFLKFFGDLQILISNEVYYICREDGVQAYQIKQVYRRSLATELIEETFGYWNRGMIVNLQHPK-HSVVRRRNLSR  198
CquiIr75e   LQQAGQLLYYRVKWIVIDRGSLDCGRFLRFFDGLKVLISSEVYYLCAGEGVDGYSIRQVYRYSIESSLAEEVYGYWYRGVVVRNDHAAIAASVRRQNLNR  159

AaegIr75e   FKLRASVVITHNETLNHLEDYREKHVDTITKKNYFLTKYLAEDLNATLKFSYVNSWGYRNPKSGKFSGMIGELQNDLADLGGTALFLTADRIKEIDYLSM  298
AalbIr75e   FKLRASVVITHNETLNHLEDYREKHVDTITKKNFFLTKYLAQDLNATLQFSYVNSWGYRDVKTGKFSGMIGELQNDQADLGGTALFLTADRIKEIDYLSM  298
CquiIr75e   FKLRASVVITHNATLNHLEDYKEKHVDTITKKNYFLTKYMIENLNATVQYSYVNSWGYRNPKTGRFSGMIGELQADQADLGATALFLTADRIREIDYLSM  259

AaegIr75e   TTPTRAKFIFRSPKLSFTDNVFLLPFSAPVWVCIISFIVLSGVLLLIIMKVELRCTNVRYGNVIRPNLMDTVMNMFGTSCQQGSYLEPKSLPAKCLILLS  398
AalbIr75e   TTPTRAKFIFRSPKLSFTDNVFLLPFSGTVWLCIISFIVLSAILLLIILKVELRCTNVRYGHVIRPNLMDTVMNMFGTSCQQGSYLEPKSLPAKCLILLS  398
CquiIr75e   TTPTRAKFVFRSPKLSVTDNVFILPFNGTVWMCIISFIVLSATLLLIIMLVEFRYTTVRYGS-FRPTLMDTMMNMFGTSCQQGSFMEPKSLPTRCLILLS  358

AaegIr75e   LIILMFLYASYSANIVALIQSPSNKIRTLEDLLNSRLETGAEDTVYNRYYFMHETEPVRKAIYDRKMKTRDGVGSSFIPLDKGVERVRQGLYAFHVELGV  498
AalbIr75e   LIILMFLYASYSANIVALIQSPSNKIRTLEDLLNSRLETGAEDTVYNKYYFMHETEPVRKAIFDRKMKGRDGSSSNFIPLDKGVERVRQGLYAFHVELGV  498
CquiIr75e   LIVLMFLYASYSANIVALIQSPSTKIRTVEDLLNSRLETGAEDTVYNKYYFMHETEPIRKALFERKMRSRDGSLDRFIPLEKGVERLRQGLYAFHVELGV  458

AaegIr75e   CYKVISETFQEDEKCGLQEIEYLNIIDPYYAVQKNSSFRELVRLSLFRLREFGIQDREHSMLYTRKPACSGGSSFIPVSIVDVWPALTLLTWASGFAGGL  598
AalbIr75e   CYKVISETYQEDEKCGLQEIEYLNIIDPYYAVQKNSSFRELVRISLFRLREFGIQGREHSMLYTRKPACSGGSSFIPVSIVDVWPALTVLSWAFAIAGGL  598
CquiIr75e   GYKVISETYQEDEKCGLQEIEYLNIIDPYYAVQKNSSFREIVRLSLFKLREFGIQGREHSMLYTKKPTCSGGSSFIPVTIVDVWPALVLLWWGFGIAAGL  558

AaegIr75e   LAGENIWARIRAKRNLA-K---VAN-GDF-CG---  624
AalbIr75e   LVGEIIWARIKVRRARA-K---VHCSG-FQFNCD-  627
CquiIr75e   VVGEFGLKKRRDIRGRFFKRSRVS--GIKPMGLS-  590
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Figure 12: Alignment of AalbIR75e with IR75e from Ae. aegypti and Culex quinquefasciatus

Gene cloning and sequencing

AalbIR8a and *AalbIR75e* coding regions were *de novo* synthesized (Twist Biosciences) and cloned into a pENTR vector. pENTR clones were then subjected to an LR clonase reaction to translocate the IR coding regions into the *Xenopus laevis*

expression vector pSP64t. Expression plasmids were purified using a GeneJET Plasmid Miniprep kit and sequenced in both directions to confirm coding region sequences.

Chemical reagents

The compounds used in these experiments were purchased from chemical suppliers (Fisher Scientific, Sigma-Aldrich) at the highest quality available. Aalb75e functional receptor responsiveness was determined using blends of biologically relevant related compounds. Chemical compounds were initially solubilized in 100% dimethylsulfoxide (DMSO) at 1M concentrations. Prior to testing, the compounds were combined into blends, based on functional group, and diluted to 10^{-4}M in ND96 buffer.

Two-electrode voltage clamping of Xenopus oocytes expressing AalbIR8a and AalbIR75e

Generation of cRNA for AalbIR8a and AalbIR75e was achieved using linearized PSP64t expression vector plasmids and the mMESSAGE mMACHINE SP6 kit (Life technologies). Stage V-VII *Xenopus laevis* oocytes were purchased from Xenopus1 (Dexter, MI, USA) and maintained in ND96 incubation media (96 mM NaCl, 2mM KCl, 5mM HEPES, 1.8mM $CaCl_2$, 1mM $MgCl_2$, pH 7.6) at 18ºC. 5% dialyzed horse serum, 50µg/mL tetracycline, 100µg/mL streptomycin, 100µg/mL penicillin, and 550µg/mL sodium pyruvate was added to the incubation media to increase oocyte survival. The oocytes were then injected with 27.6 nL of RNA using the Nanoliter 2010 injector (World Precision Instruments, Inc., Sarasota, FL, USA). Changes in current across the oocyte's membrane in response to odorants was measured using the two-microelectrode voltage-clamp technique (TEVC). To conduct our TEVC protocol, we used an OC-725C oocyte clamp (Warner Instruments, LLC, Hamden, CT, USA) set to maintain a -80mV

holding potential. When testing the effect of blends on oocytes injected with AalbIR8a and AalbIR75e, we used a blend of compounds diluted to 10^{-4}M for 8 seconds. Alterations in current as a result of blend exposure returned to baseline prior to exposure to the next blend. Data was collected using the Digidata 1550 B digitizer and pCLAMP10 software. We generated a tuning curve using 13 different carboxylic acid containing compounds including octanoic and nonanoic acid. All chemicals were administered at 10^{-4}M. Analysis of the data and figure generation were perfomed using the GraphPad Prism 8 software. Establishment of a concentration-response curve for nonanoic acids was completed using concentrations ranging from 10^{-9}M to $10^{-4.3}$M. An oocyte was exposed to each concentration for 30 seconds unless peak amplitude was achieved earlier.

Results

AalbIR75e is tuned to nonanoic acid

To investigate the responsiveness of AalbIR75e, we co-expressed AalbIR75e plus AalbIR8a in *Xenopus laevis* oocytes by injecting complementary RNAs (cRNAs) of both subunits. After a 2-5 day incubation period, responses of individual oocytes were recorded using TEVC to assess odorant-induced currents. Initial screens were performed to examine the responsiveness of the receptor complex to blends composed of biologically relevant compounds containing similar functional groups. Oocytes that expressed AalbIR75e/AalbIR8a were maximally activated by the carboxylic acid blend, which produced an approximately 10-fold greater response than any other chemical blend (Figure 13). Both IRs were necessary to achieve these responses, as oocytes lacking either protein failed to respond when stimulated with any odorant blend.

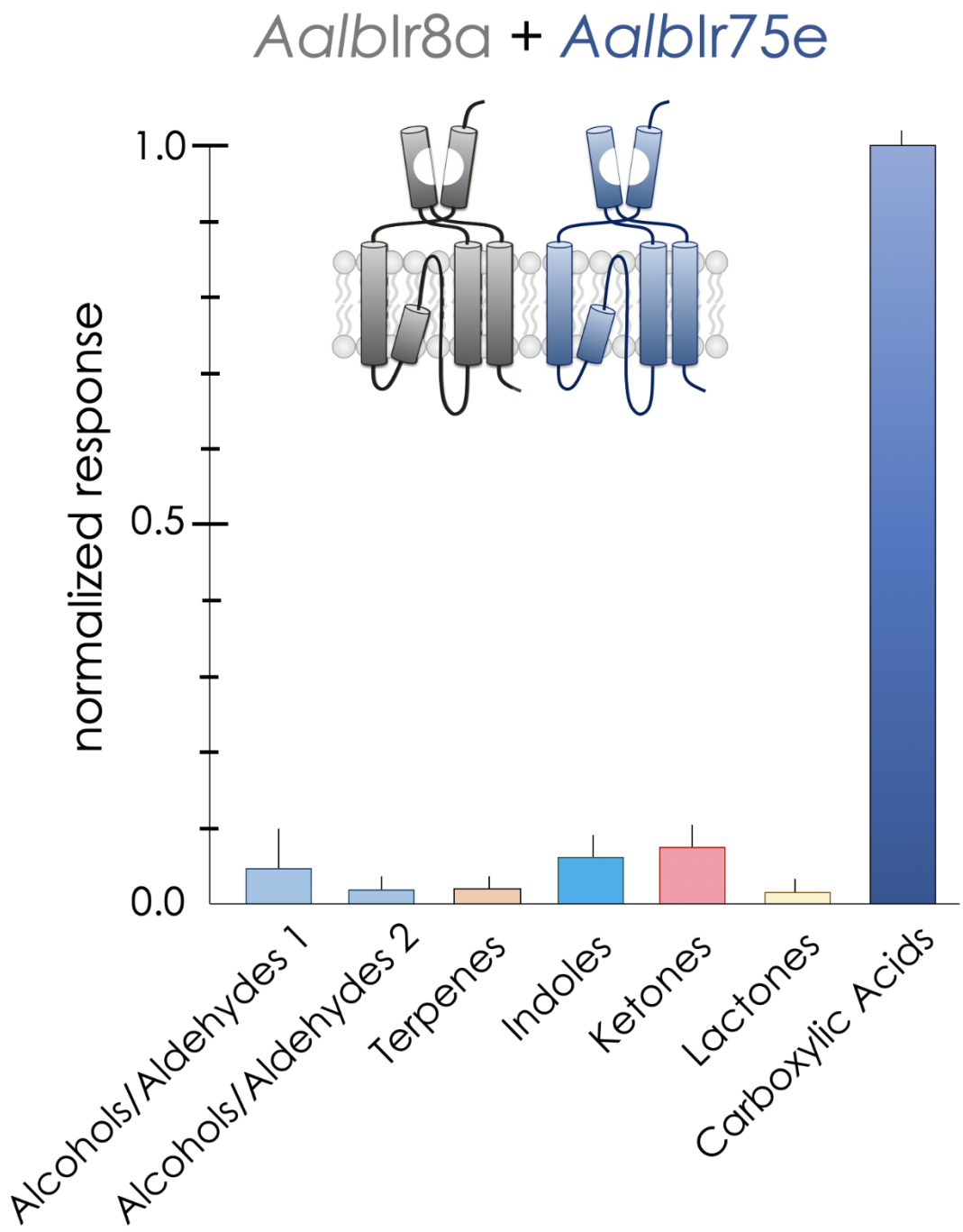

Figure 13: IR75e/8a dimer response to functional group blends

The carboxylic acids that composed the blend were then tested individually at 10^{-4}M. The AalbIR8a/AalbIR75e receptor pair responded maximally to nonanoic acid, more than a three-fold increase over octanoic acid (figure 14).

Figure 14: Response of IR75/8a dimer to selected carboxylic acids

Concentration dependency of AalbIR75e response to nonanoic acid

The response of AalbIR75e was experimentally shown to be concentration dependent. In our experiment, AalbIR8a/AalbIR75e was subjected to concentrations of nonanoic acid ranging from 10^{-9}M to $10^{-4.3}$M (figure 15).

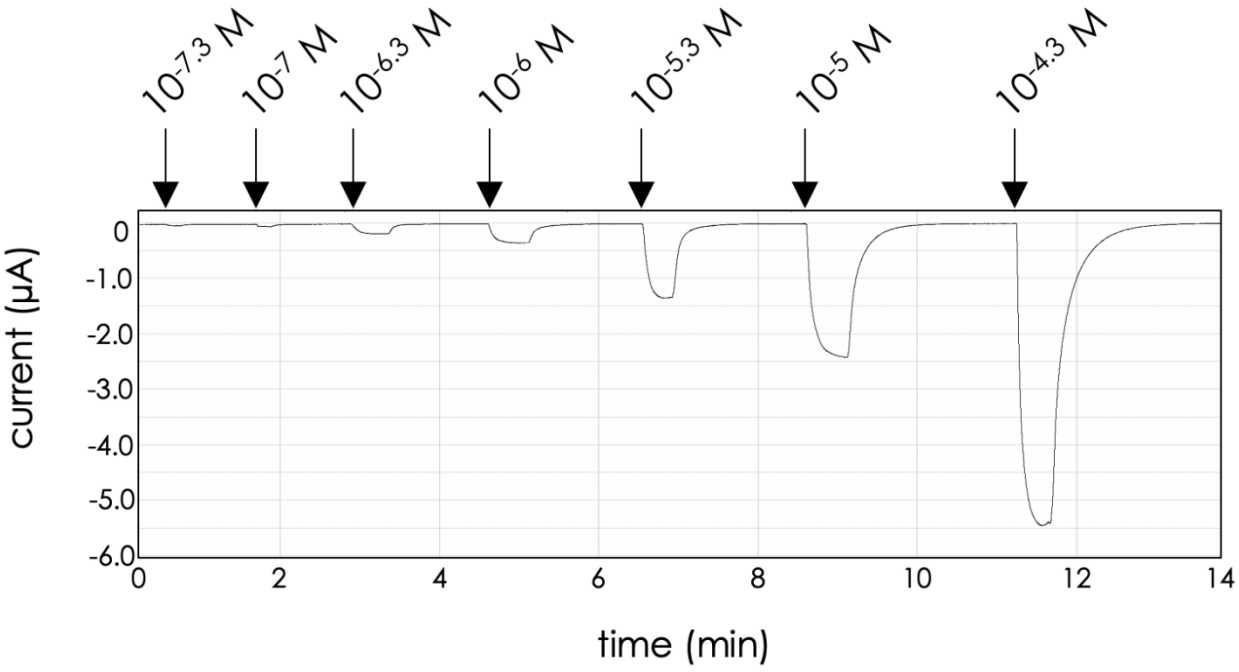

Figure 15: Concentration-response curve for nonanoic acid ranging from $10^{-7.3}$M to $10^{-4.3}$M

The average magnitude of response to concentration was plotted and a logistic regression was used to fit the points onto a sigmoidal curve. From this curve, we calculated a half-maximal effective concentration value (EC_{50}) for nonanoic acid. Nonanoic acid produced an EC_{50} value of 8.64µM (figure 16).

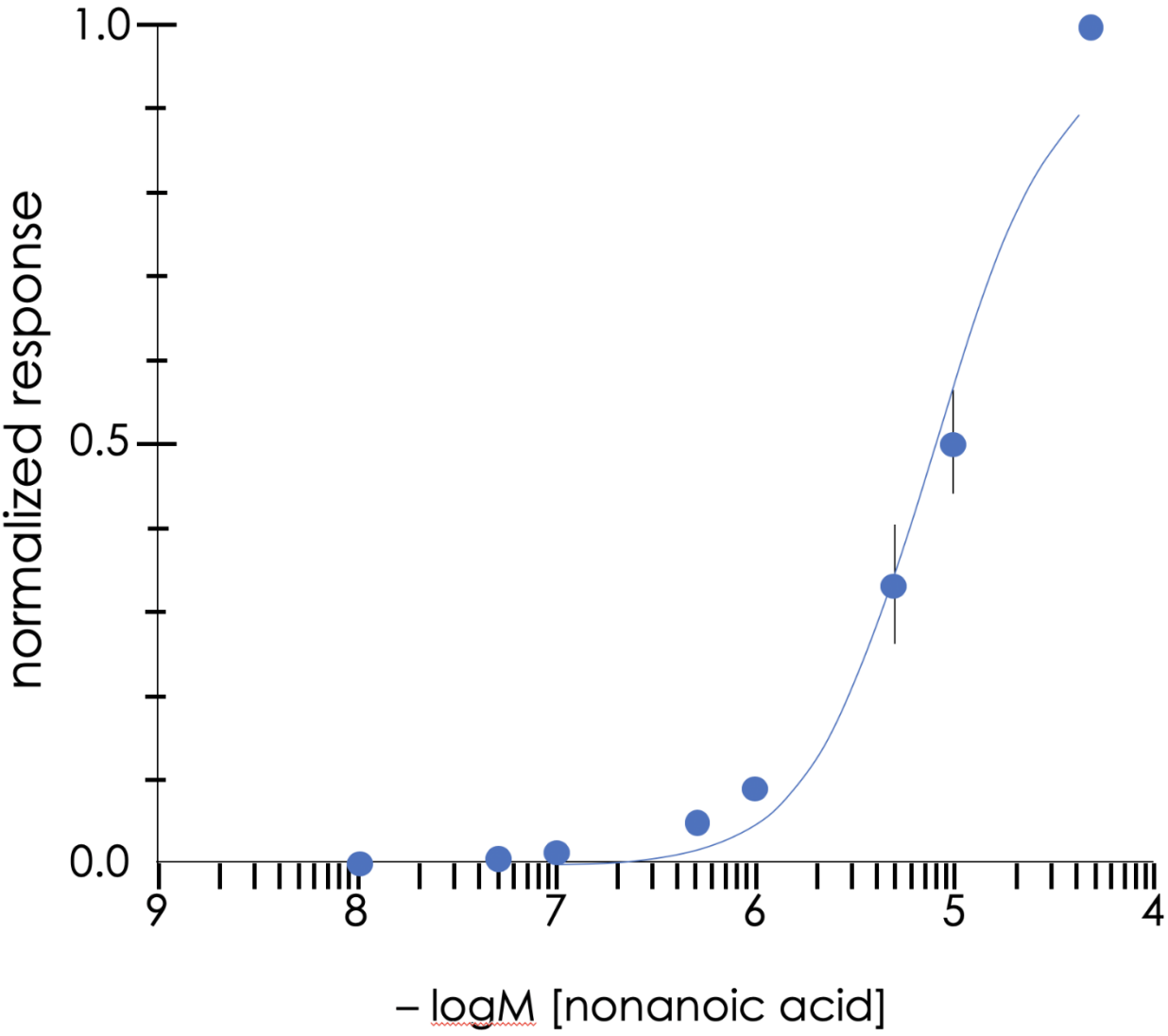

Figure 16: Logarithmic regression of responses to nonanoic acid

Discussion

Nonanoic acid is a potentially important compound relevant to host-seeking behaviors as a compound present in sweat (Bernier et al., 2000; Cork & Park, 1996; Dekker et al., 2002). Given the role of other carboxylic acids on the attraction of mosquitoes to a potential blood meal, behavioral assays to elucidate the role of AalbIR75e on host-seeking is a logical next step for research. Culex quinquefasciatus displayed down-regulation of IR75e following blood-feeding, further solidifying the idea

that IR75e plays some role in host-seeking behaviors (Taparia et al., 2017). Differential host selection between AalbIR75e^{+} mosquitoes and AalbIR75e^{-} mutants in a dual-choice olfactometer host selection paradigm could indicate that AalbIR75e is implicated in the process of host-seeking and anthropophilicity displayed by some species. Additional studies could investigate the role various mosquito repellants, such as DEET, on the detection of nonanoic acid by AalbIR75e.

Previous deorphanizations of carboxylic acids receptors in mosquitoes are scarce to our knowledge, with only IRs 75k and 75l in *Anopheles gambiae* being characterized (Pitts et al., 2017). We have demonstrated that AalbIR75e should also be considered a carboxylic acid receptor in *Aedes albopictus,* the first carboxylic acid receptor deorphaned in any *Aedes* species. AalbIR75e is highly selective for nonanoic acid and has an EC_{50} that suggests that nonanoic acid is the cognate ligand for this receptor. The deorphanization of multiple carboxylic acid receptors within the IR75 series indicates that the other receptors in this series are likely also carboxylic acid receptors. During our experiments, we realized that AalbIR75e and receptors in other mosquitoes were relatively well conserved, even across genera, suggesting that it holds an important function. One example is the conservation between AalbIR75e and AgamIR75k. When excluding the highly variable c-terminal region, these receptors are 55% identical at an amino acid level, with 73% of the amino acids possessing similar chemical characteristics. Despite the incredible similarity between these two receptors, AalbIR75e was far more selective towards nonanoic acid, as AgamIR75k displayed roughly equal affinity for octanoic and nonanoic acids. The retention of nonanoic acid sensitivity, and subsequent loss of octanoic acid sensitivity, may suggest that nonanoic acid is a more

salient host-seeking cue for mosquitoes. Additionally, AalbIR75e displayed higher affinity for nonanoic acid with an EC_{50} value of 8.64μM, compared to 35μM for AgamIR75k.

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