

OLFACTORY AND THERMOSENSORY SIGNALING IN MALARIA
VECTOR MOSQUITO *ANOPHELES GAMBIAE*

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LIST OF ABBREVIATIONS

World Health Organization	WHO
Center for Disease Control and Prevention	CDC
Odorant Receptor	OR
Olfactory Receptor Neuron	ORN
Odorant Receptor co-receptor	ORco
G Protein Coupled Receptors	GPCR
Open Reading Frame	ORF
N, N-diethyl-meta-toluamide	DEET
Transient Receptor Potential	TRP
Trans-Membrane	TM
Thermosensory TRP channels	thermo-TRPs
Phospholipase C	PLC
1, 4, 5-trisphosphate	IP ₃
Diacylglycerol	DAG
Phosphoinositide-4, 5-bisphosphate	PIP ₂
Polyunsaturated Fatty Acids	PUFAs
Small interfering RNA	siRNA
Anopheles gambiae Ionotropic Receptor	AgIR
Double strand RNA	dsRNA
Reverse Transcriptase Polymerase Chain Reaction	RT-PCR

Ribosomal protein S7	rps7
RNA interference	RNAi
Cycle Threshold	CT
Drosophila melanogaster	DM
Ionotropic Glutamate Receptor	iGluR
3-Methylphenol	3-MP
Butylamine	BA
Whole-mount Fluorescent In-situ Hybridization	WM-FISH
Fluorescent immunohistochemistry	FIHC
Alkaline phosphatase	AP
Horseradish peroxidase	HRP
Frame per second	fps
Thermotactic index	TI
Standard Error of the Mean	SEM
complementary DNA	cDNA
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid	HEPES
Activation index	AI
RNA sequencing	RNAseq
Reads Per Kilobase per Million	RPKM
Differential interference contrast	DIC
Anopheles gambiae Gustatory Receptor	AgGR
Odorant Binding Protein	OBP

Spermatheca secretory cells	SSC
Sterile Insect Technique	SIT
Innovation and Discovery in Engineering And Science	IDEAS
National Institutes of Health	NIH

CHAPTER I

INTRODUCTION: *ANOPHELES GAMBIAE* IS THE PRINCIPAL VECTOR FOR HUMAN MALARIA WHICH UTILIZE OLFACTION AND THERMOSENSATION FOR HOST SEEKING BEHAVIOR

Human Malaria and Transmission

Malaria is an infectious disease inflicted upon humans and other animals which is caused by protozoan parasites from the genus *Plasmodium*. There are at least five species of *Plasmodium* that are capable of infecting human populations¹, among which *Plasmodium falciparum* gives rise to the most severe symptoms compared to others². These symptoms include periodic high fever and shivering chills, headache, fatigue, nausea and vomiting, which could lead to dehydration, anemia, coma and even death of the individual³. In the year of 2010 alone, the World Health Organization (WHO) has estimated that malaria has killed between 660,000 and 1,200,000 people, most of whom are young children. In terms of economic loss, a clear correlation has been shown between malaria and poverty and it really contributes to a vicious cycle considering that poverty promote malaria transmission while malaria causes poverty by impeding economic growth⁴.

Human malaria is transmitted by Anopheline mosquitoes (family: Culicidae; Order: Diptera) except for the special circumstances of infections acquired trans-placentally or through blood transfusion. Approximately 460 species of *Anopheles* have been identified so far while around 70 are capable of transmitting malaria. The most efficient malaria vectors are those which prefer human population to other animals (anthropophily vs zoophily) and tend to feed and shelter indoors rather than outdoors (endophily vs exophily)⁵. Among these, *Anopheles gambiae* has been considered the most dangerous species and principal vector for human malaria due to its high degree of anthropophily with strong endophilic and endophagic traits. There are seven sibling species in the complex of which *An. gambiae* s.s. shows the strongest anthropophilic habit. A blood meal is needed to initiate vitellogenesis (egg development) and many Anophelines require at least two blood meals to pass through the pre-gravid phase⁶, thus making them even more efficient malaria vectors.

Malaria transmission starts with a blood meal of a female mosquito carrying *Plasmodium* where sporozoites are transferred to human host via the saliva and they will infect liver cells and mature into schizonts. Merozoites will be released into the bloodstream and infect red blood cells as a result of the rupture of schizonts. These blood stage parasites are

responsible for the clinical manifestations of the disease. Within the erythrocytes, the parasites undergo asexual reproduction while a proportion of parasites differentiate into sexual erythrocytic stages (gametocytes). These gametocytes are ingested by a female *Anopheles* during a blood meal and while in the mosquito's stomach, gametocytes develop into zygotes which then become motile and elongated (ookinetes). They invade the midgut wall of the mosquito where they develop into oocysts. The oocytes grow, rupture and release sporozoites, which will eventually migrate to the salivary gland of the mosquito. The sporozoites are injected into another healthy human on occasion of the next blood feeding, therefore completing the transmission cycle (Figure 1).

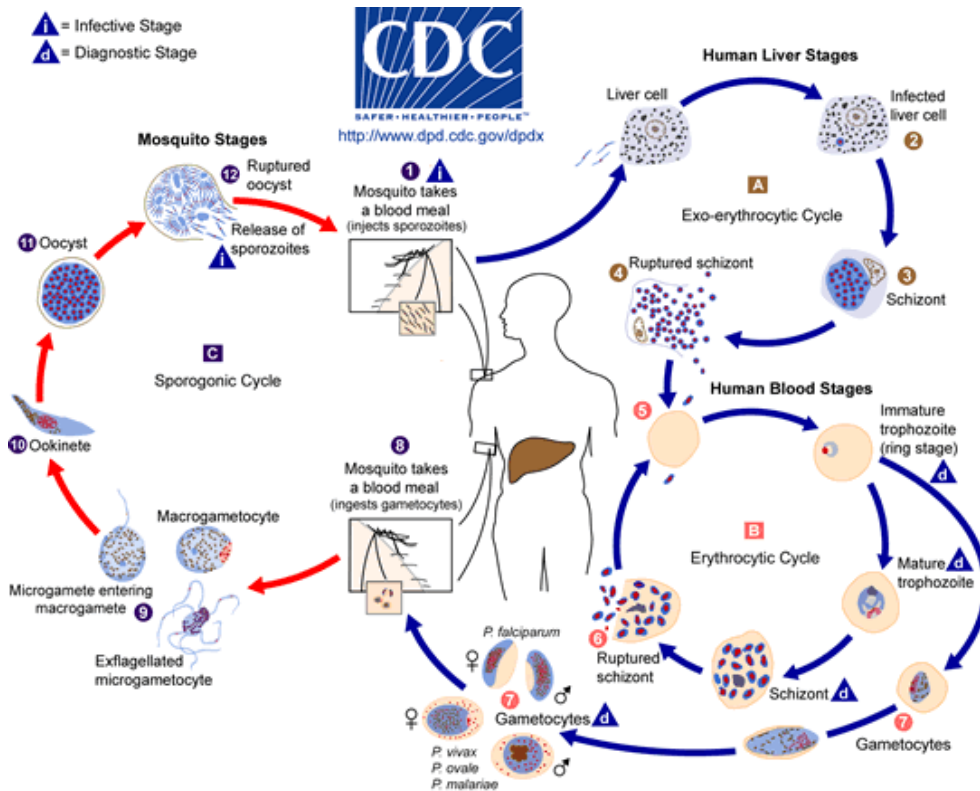


Figure 1. Transmission cycle of human malaria. Adapted from Center for Disease Control and Prevention (CDC).

Lifecycle of *Anopheles gambiae*

As is the case for all mosquitoes, *An. gambiae* goes through four stages in its lifecycle: egg, larva, pupa and adult. Adult females lay 50-200 eggs per oviposition which typically hatch in 1-3 days. Larvae live in relatively restricted aquatic habitats such as ephemeral puddles or lakes and feed on microorganisms including algae and bacteria as they develop through 4 instars, after which they metamorphose into pupae, from which adults eclose and leave the aquatic environment (Figure 2). The rate of

development and survival during the aquatic stages is greatly influenced by ambient temperatures⁷ as well as other factors such as population density and availability of nutrients⁸.

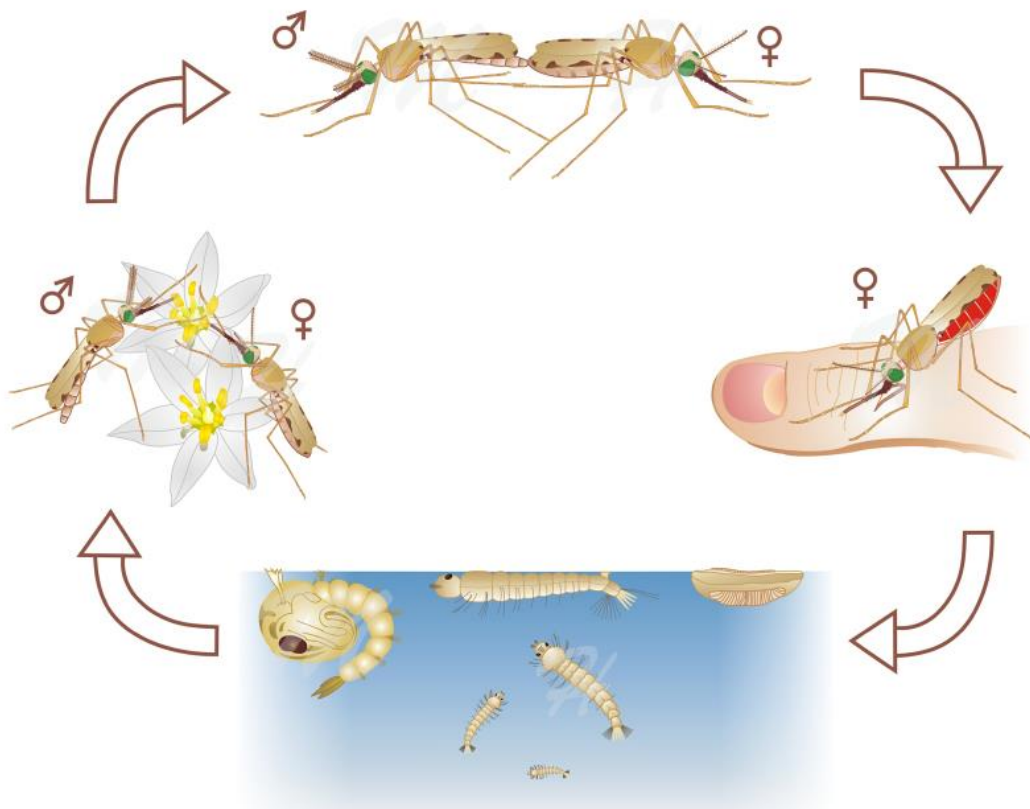


Figure 2. The lifecycle of *An. gambiae*. Adapted from www.biographix.cz. After eclosion, both female and male adult mosquitoes will feed on sugar source, mostly from flower nectar (left). Mating takes place (top) and a female *An. gambiae* will need to blood feed from a human host (right) and oviposit the eggs onto water surface (bottom).

Olfactory-mediated Host-seeking of An. gambiae

The feeding behavior of most adult female anautogenous mosquitoes include ingestion of one vertebrate blood meal during each ovarian cycle alongside consumption of plant carbohydrates⁹ (Figure 2). It is known that blood meals provide the necessary protein supplements for vitellogenesis (egg development) while sugar is the main energy reserves for a variety of mosquito behaviors such as mating and foraging⁶. *An. gambiae* take a minimum of 1 blood meal prior to oviposition during the gonotrophic cycle while studies have indicated that pre-gravid female *An. gambiae* frequently ingest multiple blood meals in this process, thus making them very efficient vectors to transmit malaria.

In order to search for a potential blood meal, female *An. gambiae* principally utilize olfaction to pinpoint the location of the human hosts¹⁰. The investigation on the chemical profile of human body odors has identified volatile sweat components that are specific to humans including carboxylic acids (E)- and (Z)-3-methyl-2-hexenoic acid and 7-octenoic acid¹¹, implying to a certain extent that how female *An. gambiae* are able to effectively distinguish between humans and other mammals. Research also has indicated that human skin microbiota plays a major role in body odor production while a high inter- and intrapersonal variation in bacteria species on human skin are identified using 16S rRNA gene sequencing,

suggesting for a possible mechanism underlying the variation in mosquito attraction between humans¹².

Volatile odor cues are peripherally recognized by chemosensory proteins including odorant receptors (ORs) which are expressed on mosquito's sensory appendages such as the antennae. This defines the onset of olfactory signaling cascade that will be further carried onto higher brain centers for processing such as the antennal lobe, mushroom body and lateral horn¹³. In this chapter, only peripheral olfaction will be discussed to elucidate the function of these sensory receptors and their roles in determining the downstream animal responses. Mosquito antenna serves as the major peripheral olfactory organ and is covered with distinct types of sensory hairs termed sensilla, each of which houses the dendrites extended from olfactory receptor neurons (ORNs) which are located at the base of sensillum¹⁴. These sensilla are distributed along 13 segments (flagellomeres) of the antenna and can be categorized based on morphological characteristics (Figure 3).

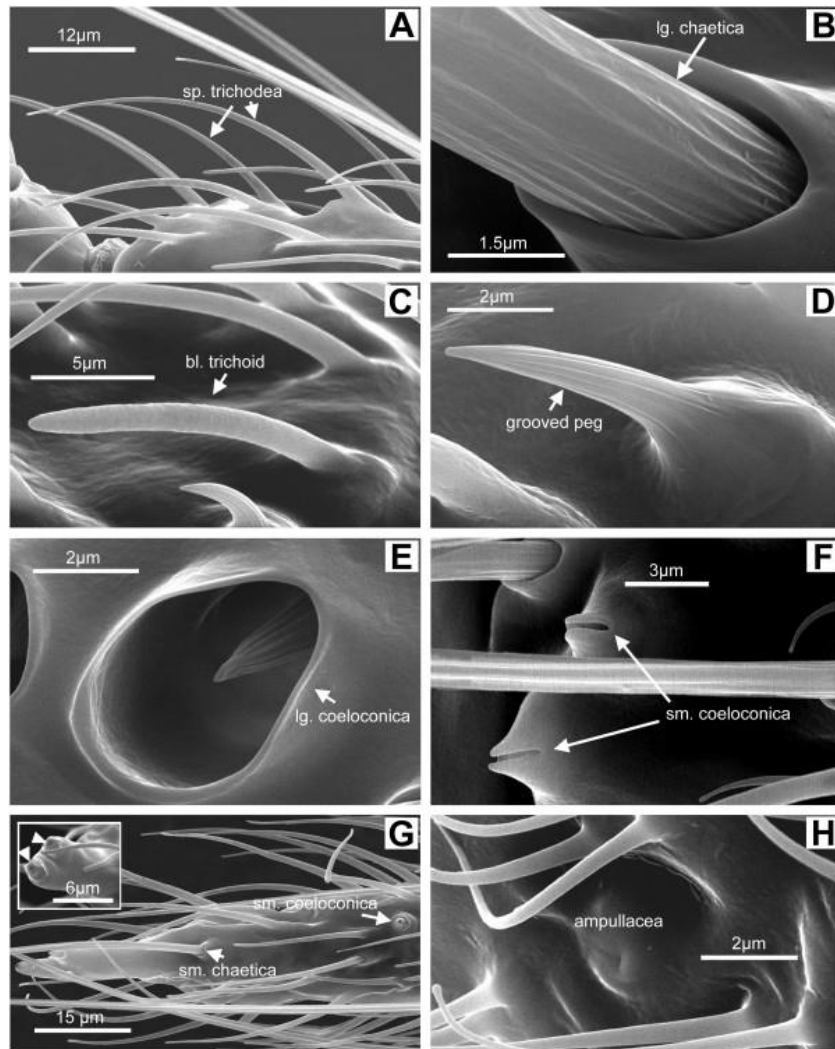


Figure 3. Sensilla types.

Adapted from Pitts. *et.al.*, *Malaria Journal*, 2006. A) Sharp trichoid sensilla have smooth surfaces, socket-less bases, and tapered ends. B) Base of a sensilla chaetica (bristle) with large socket. C) Blunt trichoid sensillum. D) Grooved peg (basiconic) sensillum. E) Large coeloconic (pitted peg) sensillum with large cuticular opening and longitudinally grooved peg set deep within. F) Small coeloconic (pitted peg) sensillum with small opening and peg not visible. G) Tip of the 13th flagellomere showing small coeloconic sensilla at the distal end (inset arrowheads) and along the surface (arrow), as well as a single small chaetica (bristle). H) Sensillum ampullaceum surrounded by microtrichia on the ventral surface of the first flagellomere.

Sensilla chaetica are sturdy bristles and occur as both large and small subtypes which are interspersed among scales on the dorsal surface. Sensilla trichodea are the most abundant among all sensilla types and can be categorized into two distinct subtypes: sharp trichodea that taper noticeably from base to tip with smooth surface as well as blunt trichodea with a round tip that is nearly as wide as the base. Sensilla basiconica is also known as grooved pegs and appear as thorn-shaped hair with 10-12 grooves on the surface. Sensilla coeloconica are small, thick-walled sensilla that houses a peg set into the bottom of a pit whose tips often project to below the external rim of the socket. Additionally, sensilla ampullaceal are also small, thick-walled peg set at the bottom of a tube. However, unlike coeloconic sensilla, the pegs project perpendicular to the tube walls. Sensilla trichodea are confirmed to be the major olfactory chemosensilla where they respond to olfactory stimuli either by an increase or decrease in impulse frequency as compared to the spontaneous spiking activity. It was shown as early as 1968 that sharp trichodea are sensitive to host odors such as those from a human hand while the blunt trichodea are sensitive to vapors of commercial repellents¹⁴, confirming their roles in sensing environmental odors.

Throughout four decades of research on insect chemosensilla, extensive knowledge is known for the molecular, anatomical and

functional organization of the insect olfactory system, particularly in *Drosophila melanogaster*¹⁵. Olfactory receptor neurons (ORNs) are located at the base of each olfactory sensillum and project their axons centrally to synaptic modules in the brain named glomeruli. The odorant molecules are recognized by the ligand-binding members of the olfactory receptor (OR) along with a highly conserved and broadly expressed co-receptor ORco which are expressed in both the ORNs as well as the dendrites extended from these neurons¹⁶. The insect ORs were first identified using a novel search algorithm to probe the *Drosophila* genome for predicted 7-transmembrane G-Protein Coupled Receptors (GPCRs) with longer than 300bp Open Reading Frame (ORF) based on statistically examining the physicochemical profile¹⁷. The search criteria was chosen due to the fact that vertebrate ORs were confirmed to be GPCRs, however, insect ORs show opposite topology when compared to mammalian ORs and they share very little sequence homology and are also evolutionarily divergent from each other as no common ancestor can be identified using bioinformatics approaches¹⁸.

Recent research has revealed the ionic conductivity of the insect ORs and they are now more and more believed to be ligand-gated ion channels other than GPCRs although there is evidence that a slow-responsive metabotropic second-messenger pathway is present additional

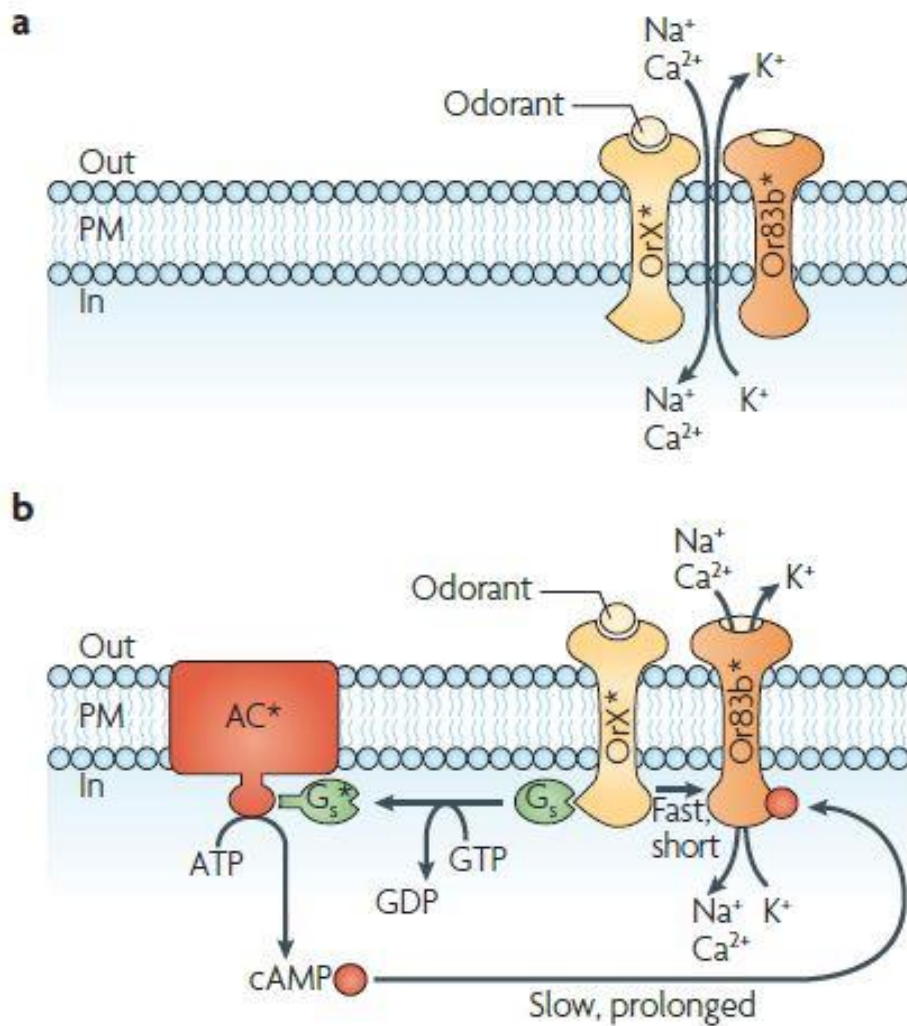


Figure 4. Models of olfactory signaling cascades in insects. Adapted from Kaupp. *Nature Reviews*, 2010. a) ORs from heteromeric ion channels that are directly gated by odorants. The channel complex is comprised of ligand-binding ORX as well as ORco. The channel pore of this model is not specified although current research indicates the pore region is contributed by both ORX and ORco subunits¹⁹. b) In addition to the channel model, the ORco subunit is also linked to a metabotropic pathway that is presumably slower but with amplification of signal through a second-messenger pathway the response is much stronger and prolonged. In this model it is proposed only ORco contributes to the channel pore, not ORX.

to the fast-responsive kinetics of ligand gating (Figure 4)^{20,21}. The ORco and ORX will form a heteromeric ion channel complex on the dendritic membrane with unknown stoichiometry. Upon the binding of odorant to ORX, the channel pore will open to allow influx of cations, which in turn, leads to membrane depolarization and eventually an action potential¹⁶. Thus the electric signal of the odorant can be transmitted downstream. To date, 79 ligand-binding *Ors* and 1 *Orco* gene have been annotated in *An. gambiae* genome and numerous studies have validated the essential role of ORs in a comprehensive array of olfactory-directed behaviors in mosquitoes including mating, nectar feeding, host seeking and oviposition, all of which are vital for the survival of the species¹⁰. The OR-mediated peripheral olfaction has been shown to mediate host selection as well as sensitivity to the insect repellent N,N-diethyl-meta-toluamide (DEET) and one of the recent studies demonstrate that the *orco* mutant *Ae. aegypti* fail to discriminate between human and animal odors, therefore strengthening the indispensable role of ORs in determining the host seeking behavior of mosquitoes²².

Larval olfactory system

Previous studies have been carried out to examine the molecular basis of olfaction in insect larvae, due to their relatively simplified neuronal

circuits and convenience to conduct behavioral analysis²³. In *An. gambiae* 4th instar larvae, their major olfactory organ has been identified, which is an aporous cone-shaped structure located at the distal tip of the antennae named sensory cone²⁴. As confirmed by both RT-PCR and In-situ hybridization assays, there are 12 tuning ORs expressed on the larval antenna. The sensory cone is innervated by dendrites extended from a cluster of 12 ORNs with each expressing a combination of *AgOrco* and *AgOrX*. *In vitro* heterologous expression of larval ORs in *Xenopus* oocytes have demonstrated their efficacy to respond to a range of natural and synthetic odors. The combinatorial odor coding for larval ORs is also investigated by screening them against a panel of odorants. The response spectrum of any given larval OR is discrete such that some ORs (AgOR1, AgOR34) respond to a narrow set of odorants while others (AgOR10, AgOR40) are much more broadly tuned. On the other hand, a single odorant is also able to elicit responses from multiple larval ORs with varying amplitude. For example, acetophenone, a volatile aromatic ketone emitted by plants, which was suggested to be an insect attractant²⁵, generate electric responses from AgOR6, AgOR10, AgOR28 and AgOR37²⁴. Behaviorally, the 4th instar *An. gambiae* larvae exhibit dose-dependent locomotion towards/away from several odorants which are shown to activate larval ORs in the heterologous expression system.

Additionally, in chapter 2, I will provide a detailed description of a more recent study that was carried out on late-stage *An. gambiae* larvae to elucidate the *in vivo* role of peripherally expressed ORs in directing larval responses towards odor stimuli as well as the characterization of a distinct peripheral signaling pathway that is independent of ORs, but mediated by a gene from the family of Inotropic receptors (IRs) that are distantly related to glutamate receptors.

Expression of ORs in Non-chemosensory tissue

Although odorant receptors are, judged by the name, primarily studied in the peripheral chemosensory organs that mediate perception of environmental odorants, it also has been documented that the expression of ORs is not restricted to the olfactory system. In mammals, ORs have been found outside the olfactory system, which are suggested to function in skeletal muscle development, regeneration, human sperm chemotaxis etc. Meanwhile, the transcript for human ORs have been “ectopically” identified in many internal organs, to name but a few, heart, spleen, pancreas, placenta, lung, kidney. However, the possible roles of these cryptic OR expression are to a large extent, mysterious²⁶. As for insects, very few attention has been given to the olfactory receptors in tissues other than the chemosensory appendages. Nevertheless, an RNA-

sequencing-based survey of OR expression level between the whole body of male and female *An. gambiae* has revealed a number of *Ors*, including *Orco*, show male-biased expression pattern. While in samples containing only antennae or maxillary palps, respectively, which are the major chemosensory organs in mosquito, these ORs are undoubtedly enhanced towards female mosquitoes²⁷. These results have indicated that transcript for ORs are present in tissues other than the peripheral sensory appendages within the male *An. gambiae*. In chapter 4, I will expand upon this discovery and describe the expression for a variety of ORs in the male testis while these ORs are suggested to be functional in mediating chemical-induced activation of flagellar movement in mature spermatozoa.

Thermal Sensitivity in Host-seeking

In addition to olfaction, female *An. gambiae* utilize body heat emitted from human host as a relatively short-range guidance and it has been shown from multiple studies that heat sensitivity is able to synergize with olfaction to enhance the efficiency of landing behavior in mosquitoes²⁸. Heat cues are common to all warm-blooded hosts so it is not likely that body heat alone will play a major role in directing human preference in *An. gambiae*²⁹, however, basic study on mosquito thermosensation would undoubtedly shed light in the design of novel

control approaches that interfere with mosquito's host seeking ability as well as serve as a new molecular target for insecticide development as thermal sensitivity is also vital for the survival of poikilothermic insects that are incapable of maintaining thermal homeostasis.

Transient Receptor Potential (TRP) Channels in Thermosensation

The first TRP gene was discovered in 1969 when Cosens and Manning characterized a *Drosophila* mutant that showed a transient instead of a sustained response to bright light³⁰. The photoreceptor cells in the mutant flies with sustained light exposure displayed a transient, rather than normal plateau-like receptor potential. Thus the mutant gene gained its name *trp*, abbreviated for Transient Receptor Potential. Not until 20 years later that *trp* gene was cloned and revealed to function as a Ca²⁺-permeable cation channel³¹. To date, research on TRP channel genes have revealed 28 members in human³², 16 in flies³³ and at least 10 in *An.gambiae* (unpublished data). They can be sub-categorized into 7 subfamilies namely TRPC, TRPV, TRPM, TRPA, TRPP, TRPML and TRPN based on sequence similarity³². The TRPC subfamily ("C" stands for canonical) comprises of proteins with highest homology to the *Drosophila* TRP protein, hence gained its name. The other subfamilies were named after their first identified members: the TRPV subfamily after

the vanilloid receptor 1 (*trpv1*), the TRPM subfamily after the tumor suppressor melastatin (*trpm1*), the TRPA subfamily after the protein denoted ankyrin-like transmembrane domains 1 (*trpa1*), TRPN after the no mechanoreceptor potential C (*nompC*) gene from *Drosophila*, TRPP after the polycystic kidney disease-related protein 2 (*trpp2*), and TRPML after mucolipin (*trpml1*)³⁴.

TRP channels are found to be expressed in a broad spectrum of organisms from worms to human and they all share 6 trans-membrane (6-TM) domains, yet characteristic structures including ankyrin repeats, coiled coil domain, and protein kinase domains are specific to certain subfamilies. The TRP super-family of membrane proteins displays distinct ion selectivity, modes of action and physiological functions among different subfamily and even members within the same subgroup. TRP channels were initially considered to be PLC-dependent or a Ca²⁺ store-dependent cation channel until an expression-cloning method was utilized to isolate a vanilloid receptor, which displayed high identity with *Drosophila* TRP. This receptor, subsequently renamed TRPV1, was activated not only by vanilloids such as capsaicin, but also temperatures above 43°C³⁵. The investigation of thermosensory TRP channels (thermo-TRPs) has led to the characterization of no less than 6 thermo-TRPs in mammals and an equal number in insects.

In *Drosophila*, the thermo-receptors are housed in the third antennal segment according to ablation studies³⁶ while in *An. gambiae*, a pair of small coeloconic sensilla on the distal tip of adult female antennae were shown to contain neurons that specifically respond to rise of temperatures³⁷. In contrast with limited achievements regarding peripheral thermo-receptors in mosquitoes, comprehensive studies have been carried out in *Drosophila* on thermo-TRPs in the past two decades. *painless*, a member of TRPA subfamily, has heat sensitivity that is essential for avoidance of noxious heat (above 42°C), thus uncovering its role as a primary noxious heat detector in *Drosophila*³⁸. The other gene in *Drosophila* TRPA subfamily, *pyrexia*, is activated approximately at 40°C and holds high potassium permeability. In addition, evidence exists that activation of *Pyrexia* is essential for the prevention of paralysis during high temperature stress³⁹. A third member in TRPA subfamily, *trpa1*, is one of the best-studied thermo-TRPs that mediates med-high temperature sensation which is activated above 25°C and contributes to avoidance of sub-lethal warmer temperatures in *Drosophila*⁴⁰. TRPA1 possesses at least two distinct functions *in vivo* as TRPA1 is involved in sensing electrophiles in gustatory neurons and derives behavioral avoidance to several tissue-damaging chemicals. It also has been shown that

invertebrate and vertebrate TRPA1 share a common ancestor and critical characteristics required for electrophile detection⁴¹.

TRP channels that are involved in photo-transduction in mammals and flies utilize a phospholipase C (PLC)-mediated signaling cascade (Figure 5). The activation of PLC leads to the production of inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol (DAG) as well as the release of Ca²⁺ from internal IP₃-sensitive stores. They are either positively or negatively regulated by second messengers derived from the hydrolysis of phosphoinositide-4,5-bisphosphate (PIP₂) or products from these second messengers⁴².

In terms of thermo-TRPs, recent behavioral studies on *Drosophila* larvae regarding their thermo-preference in a thermo gradient indicated the larvae required PLC β to distinguish between optimal temperature (18°C) and suboptimal temperature, thus suggesting dTRPA1 functions through a PLC-mediated GPCR pathway⁴³. It is more recently discovered that Rhodopsin is involved in *Drosophila* thermosensation, thus uncovering a novel role for this canonical visual G-protein as well as strengthening the view that a second-messenger pathway is employed in thermal sensitivity of insects⁴⁴. Furthermore, the implication that activation of TRPA1 is conjugated to a signaling cascade may promote signal amplification of small differences in environmental temperature to facilitate

adaptation within the comfortable range. Therefore, thermosensation through TRP channels may echo the signaling transduction adapted by photo-transduction during the course of evolution.

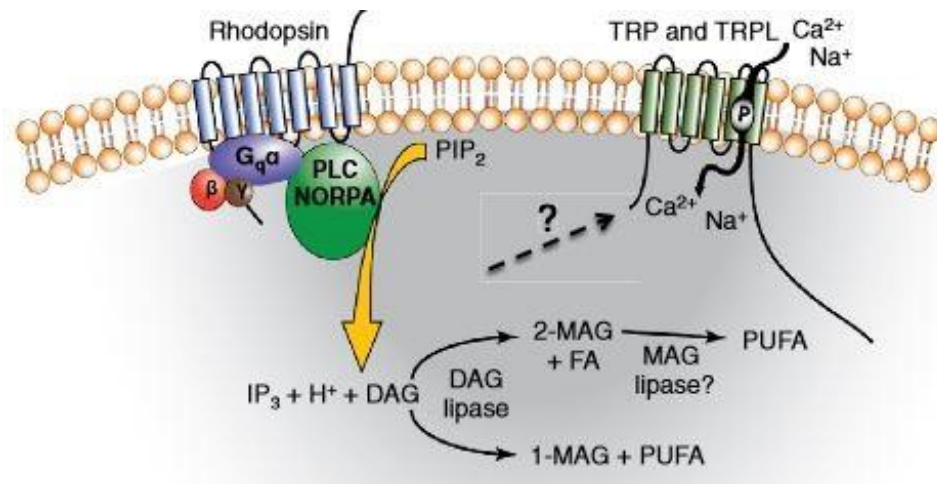


Figure 5. Model of TRP channel-mediated *Drosophila* phototransduction. Adapted from Fowler *et.al.*, *Life Sciences*, 2013. Capture of photon by the membrane-bound Rhodopsin initiates a G-protein coupled signaling cascade that the effector protein PLC encoded by the gene *norpA* will catalyze the cleavage of membrane-bound PIP₂, leading to the production of IP₃, DAG and a proton. It is still in debate as to the mechanisms underlying the activation of TRP channel following the hydrolysis of PIP₂. Current opinions regarding the gating of TRP channels are diversified. It is suggested that either polyunsaturated fatty acids (PUFAs) through metabolism of DAG activates TRP channel or acidification combined with a decline in inhibitory PIP₂ gates the channels.

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CHAPTER II

DISTINCT OLFACTORY SIGNALING MECHANISMS IN THE MALARIA VECTOR MOSQUITO *ANOPHELES GAMBIAE*

Preface

This following article was published in the journal of *PLoS Biology* in 2010 (Volume 8, No. 8, pii: e1000467). I was a co-first author on this paper along with other co-authors including R. Jason Pitts (co-first), Johnathan D. Bohbot (third author), Patrick L. Jones (fourth author), Guirong Wang (fifth author) and Laurence J. Zwiebel (corresponding author). In this paper, I have developed a novel single-larval bioassay for the purpose of quantifying olfactory-driven behaviors when they are challenged with a series of natural or synthetic chemicals which are able to elicit larval responses *in vivo*. I have also implemented an RNAi-based gene silencing protocol in this paper to specifically knockdown genes via injection of small interfering RNA (siRNA) on 3rd instar larvae to evaluate the effect of peripheral olfactory genes in downstream larval responses. I took a leading role in experimental design, data acquisition, statistical analysis as well as figure and manuscript preparation. I want to thank my co-first author R. Jason Pitts for his contribution in the annotation of *An.*

gambiae Ionotropic Receptors (*AgIRs*), Johnathan D. Bohbot for figure design, Laurence J. Zwiebel for his support and discussions on the experimental data. I have modified the gene name for *Or83b* or *Or7* from the original publication to *Orco* to conform to the recent change of nomenclature in the field¹.

Introduction

Chemosensory cues play a central role in directing much of the behavioral repertoire and are a significant determinant in the vectorial capacity of female *An. gambiae* mosquitoes, which are responsible for the transmission of human malaria². Significant progress has been made in identifying the components of olfactory pathways in *An. gambiae*³⁻⁷. Nonetheless, there is a paucity of information regarding the precise molecular mechanisms that mediate olfactory signaling in *An. gambiae*.

At the center of the peripheral olfactory signal transduction pathway in *An. gambiae* is a family of odorant receptors (AgORs) that are selectively expressed in olfactory receptor neurons (ORNs). Although originally identified as candidate G-protein-coupled receptors (GPCRs)⁸, several studies have disputed the GPCR nature of Anopheline and other insect ORs⁹⁻¹², which likely form ligand-gated heteromeric ion channels that activate ORNs through ionotropic as well as perhaps metabotropic

mechanisms. In addition, members of a family of another set of chemosensory receptors related to ionotropic glutamate receptors have recently been described in *Drosophila melanogaster*¹³.

The majority of insect ORNs typically express at least two ORs that are likely to form complexes of undetermined stoichiometry that are composed of one highly conserved non-conventional ORco-like protein together with a conventional OR that presumably mediates odorant binding specificity^{5,9,14}. In *An. gambiae*, 73 of the 79 AgORs originally identified are expressed in the adult and 13 are expressed in larval stages¹⁵. The non-conventional Anopheline ORco-like family member, AgORco, is widely expressed in nearly all olfactory sensilla with the notable exception of grooved-peg sensilla⁶, which are activated *in vivo* by compounds such as ammonia, lactic acid, and other carboxylic acids that are major components of human sweat^{16,17} known to evoke physiological and/or behavioral activity in *An. gambiae*^{18,19}. Indeed, recent functional analyses of AgOR odor space reveal a paucity of responses for these groups of odorants, suggesting Anopheline sensitivity to amines and other variant odorants may lie outside of AgOR-based signaling.

In order to improve our understanding of mosquito olfaction, we have continued to utilize the relative simplicity of the *An. gambiae* larval olfactory system, which consists of only 12 ORNs¹⁵. In previous studies

utilizing behavioral and functional approaches to describe the molecular and cellular basis for olfactory responses to a range of natural and synthetic chemical stimuli, we identified a subset of AgORs expressed in the larval antenna that are tuned to odorants that elicit specific behavioral responses. Building upon those studies, we now use RNAi-based gene-silencing approaches to validate in vivo the role of AgORs in larval olfactory signal transduction and specifically identify the molecular receptor that mediates the repellent activity of N, N-diethyl-m-toluamide (DEET). In addition, we have identified and characterized a family of chemosensory receptors that are related to inotropic glutamate receptors (AgIRs) that underlie a novel-signaling pathway that is independent of AgOR activity. We propose that *An. gambiae* expresses distinct signaling pathways that participate in larval olfaction and are likely to also be active in mediating adult responses to a diverse range of chemosensory stimuli. These studies further our understanding of the molecular basis of olfaction and olfactory-driven behaviors in *An. gambiae* and lay the foundation for advancing alternatives to mosquito control strategies focused on adult life stages.

Materials and Methods

Mosquito Rearing

An. gambiae sensu stricto, originated from Suakoko, Liberia, was reared as described³. For stock propagation, 4- to 5-d-old female mosquitoes were blood fed for 30–45 min on anesthetized mice, following the guidelines set by Vanderbilt Institutional Animal Care and Use Committee.

Individual Larval Behavioral Assays

Larval assays were conducted between ZT2 and ZT10 during the standard LD12:12 rearing cycle. Here, *An. gambiae* 3rd or 4th instar larvae were removed from rearing pans, rinsed carefully with distilled water to eliminate any remaining food residue, and kept in segregated containers with distilled water for 30 min. Odorant stocks were made by dissolving odorant (>99% pure or of the highest grade commercially available) in pre-heated (70°C) 2% NuSieve, GTG low-melting-temperature agarose (Cambrex Bio Science). The assay was performed in a 10×1.5 cm Petri dish containing 50 ml of 27°C distilled water. The odorant and larva dropping spots were located at opposite ends along the diameter and marked by a solid circle and a cross, respectively. The odorant/control

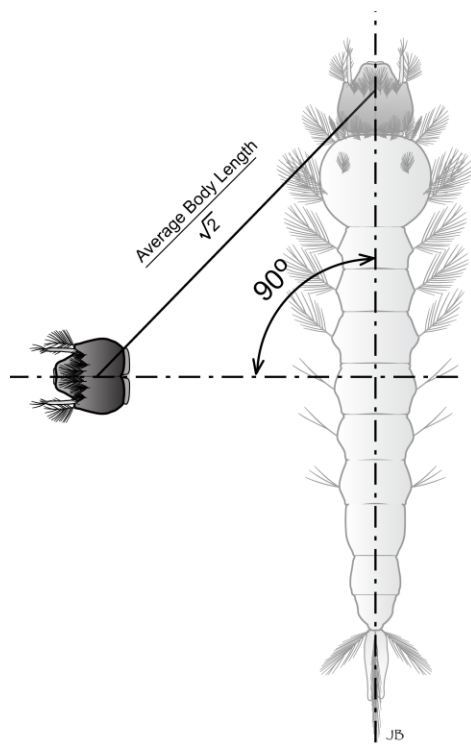
stock was placed into the dish for 1 min beforehand to equilibrate, and the larva was gently introduced at the marked spot.

Real-time images of larval movements were obtained and downloaded at 1 s intervals for the duration of the 5 min assay using a custom-designed 30 frames/s video camera/computer/software system (Model NC-70, DAGE-MTI, Michigan City, Apple PowerMac 8500/Scion Image J v1.63, National Institutes of Health, USA). At the conclusion of each assay, all larvae were individually stored at -80°C for molecular analyses, as described below. The images were subsequently sorted and analyzed using Image J (version 1.40g, NIH, USA) with its Mtrack J plug-in (version 1.3.0). The analysis of larval responses was carried out by tracking the motion of individual larva after marking the position of the larva's anterior, which was easily discernable in our system. In this manner, we were able to monitor and calculate the number of larval turns, overall movement, resting time (s), and average velocity (mm/s) to provide a comprehensive characterization of larval behavior patterns. Similarly, a turn threshold was defined such that if the intersection angle between two successive larval tracking vectors exceeded 45° , the larvae were considered to have carried out a turn (Figure 1). Similarly, movement thresholds were defined so as to recognize false movements and account for the tendency of *An. gambiae* larvae to stochastically perform body

swirls that appear to lack any horizontal locomotion. In our hands, a movement threshold was set by establishing that an individual larva turns 90° relative to an axis set at the body-length midpoint; the distance between the previous and the current position of the larval head can be calculated using the equation: $\text{body length}/\sqrt{2}$. By setting the movement threshold in such a manner, we were able to compensate for false movements that result from the tendency of *An. gambiae* larvae to stochastically perform body swirls that appear to lack any horizontal locomotion. After measurement of multiple ($n > 30$) stage-4 larvae, we calculated the average larval body length as ~3.25 mm in our CCD system, thereby establishing a threshold for larval movements at ~2.3 mm, such that any shift in larval head position exceeding this value was defined as a single instance of larval movement (Figure 1). In addition to analyzing tracking data for the number of movements and turns, we also measured the average velocity (mm/s) and resting time (s) over the course of the entire assay. Arithmetic means for each assay/treatment were analyzed for statistical significance using single-factor ANOVA; significant results were followed up with Tukey-Kramer post-tests to distinguish among groups using JMP software (v. 4.0.4, SAS, Cary, NC). In the cases where antennal and maxillary palp ablations of larvae were conducted, all manipulations were carried out by manual dissection at 3rd instar stages,

after which larvae were allowed to recover for 24 h prior to behavioral testing.

A



B

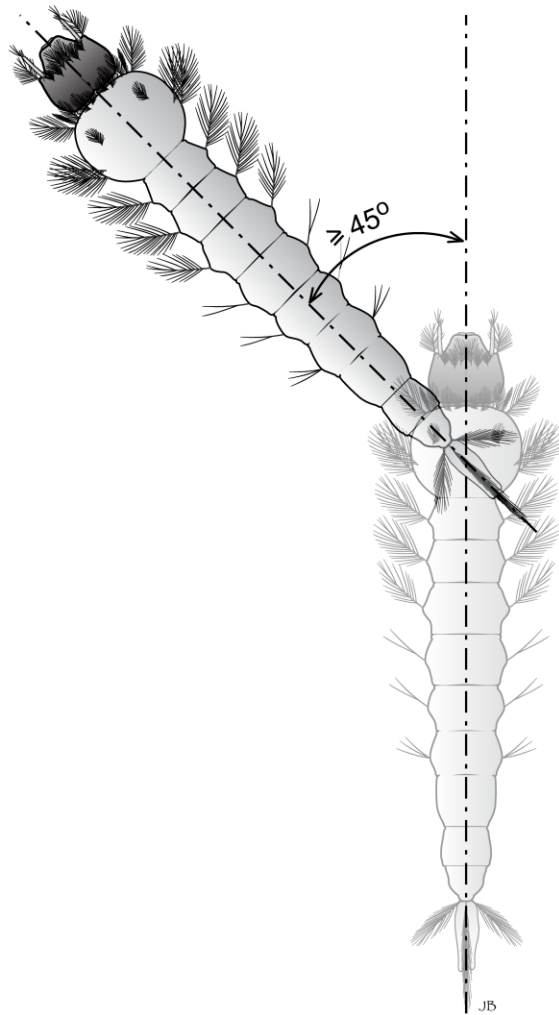


Figure 1. Operational definitions of larval movements and turns.

(A) A larval body movement threshold is characterized by a larva turning its body axis by 90° and its head traveling the distance indicated. (B) A larval turn threshold is defined by a 45° angle between two successive larval tracking vectors.

AgIR Identification and Expression

Candidate AgIR sequences were identified in both the *An. gambiae* genome using DmIR amino acid sequences as tBLASTn and BLASTp queries, respectively. Potential exon-intron gene models were predicted based on homology to DmIRs or AgIRs, as well as with the aid of a Hidden Markov Model-based gene structure predictor (www.Softberry.com). Iterative searches of all gene models were carried out until no new candidates were identified. Conceptual translations of full AgIR coding sequences were aligned with DmIR protein sequences using Clustal X. Phylogenetic trees were constructed using the Neighbor-Joining method²⁰ with bootstrap resampling of 1,000 pseudo-replicates. Transmembrane helices were predicted using Hidden Markov Model-based software from the Center for Biological Sequence Analysis (<http://www.cbs.dtu.dk/services/TMHMM-2.0/>). Antennae from late-instar *An. gambiae* larvae were hand-dissected into RNALater-Ice solution (Ambion, Austin, TX). Total RNA extraction and cDNA synthesis were performed using the RNeasy Mini (Qiagen) and Transcriptor First Strand cDNA Synthesis (Roche) kits, respectively. Antennal cDNA was used as a template in PCR as described³. PCR primers specific for *AgIrs* were as follows: *AgIr8a*: 5'-CCCTATGAGTGCAGAAAATT-3' and 5'-GGTACAGCACGTCTTCTGCG-3'; *AgIr25a*: 5'-

CAACCGACATACGCTACCAA-3' and r5'-ACGATGAATACGCCTCCGAT-3'; *AgIr41a*: f5'-ACTGGGAACTGGAGGTGGTG-3' and r5'-CTAAGGTGTCTCACTCCTCC-3'; *AgIr41n*: f5'-ATGCACGATACATCTTGCCG-3' and r5'-TAAAGGACAGGAACGGTGTG-3'; *AgIr76b*: f5'-CACGCTCCCAATCAACAATG-3' and r5'-GATGGCGGCTAAACACTTCC-3'; *AgNMDAR2* f5'-AAGTTGGTGCTATGGATCAT-3' and r5'-ACACCATACGCATATACCCG-3'; *rps7*: f5'-GGCGATCATCATCTACGTGC-3' and r5'-GTAGCTGCTGCAAACCTTCGG-3'. cDNA amplicons were TOPO-TA cloned into plasmid pCRII (Invitrogen) and sequenced to confirm their identities.

siRNA Preparation and Injection

Double-stranded (ds) RNAs against a specific target gene were prepared and purified using bidirectional *in vitro* transcription of full-length cDNA templates using flanking T7 transcription initiation sites, and siRNAs were prepared via RNaseIII digestion using Silencer siRNA Construction reagents and protocols (Applied BioSystems/Ambion, Austin, TX). Healthy, wild-type 3rd instar *An. gambiae* larvae were chosen for micro-injection. They were pre-immobilized on 3mm filter paper on top of a 4°C

chill platform (BioQuip Inc, Rancho Dominguez, CA). Additional desiccation was achieved using Kimwipes (Kimberly-Clark, Dallas TX) to gently dry individual larva. Twin styrofoam strips were also employed as temperature sinks to reduce distress from cold temperatures. Single barrel borosilicate glass capillary pipettes (World Precision Instruments, Sarasota, FL) were pulled (using a P-97 puller, Sutter Instruments, Novato, CA) and beveled (using a Narishige EG-5 beveller, Tokyo, Japan) to form microinjection needles. For larval microinjection, 27.6 nL of 100 nM siRNA were injected into the dorsal side of the larval thorax using a Nanoliter 2000 system (World Precision Instruments, Sarasota, FL). Post-injection, larvae were allowed to recover in 27°C distilled H₂O with 1 ml of larval food (as described in Mosquito Rearing section) for 48 h. Larvae were monitored every 24 h post-injection, and non-viable individuals were discarded.

Real-Time PCR (qRT-PCR)

Subsequent to experimental treatments and behavioral assays, *AgOrco*, *AgOr40* and *AgIr76b* transcript levels were determined by means of quantitative RT-PCR. Each sample was comprised of 10 (*AgOrco*) or 30 (*AgOr40*, *AgIr76b*) larval heads that were hand-dissected from batches of control and experimental *An. gambiae* larvae. RNA extraction and cDNA

synthesis were performed using the QIAGEN RNeasy Mini Kit and Roche Transcriptor First Strand cDNA Synthesis Kit, respectively. All primers in the assay were designed to span predicted introns in order to distinguish well between genomic DNA and cDNA templates. *An. gambiae* ribosomal protein S7 (*rps7*), which is constitutively expressed at high levels in all tissues, was chosen as control gene to measure the relative levels of mRNA of target genes in vivo. Primer sequences are as follows: *rps7*: f5'-GGCGATCATCATCTACGTGC-3' and r5'-GTAGCTGCTGCAAACCTTCGG-3' (product size: 458bp cDNA); *AgOrco*: f5'-ATCTTTGGCAATCGGCTCATC-3' and r5'-GGCTCCAAGAACCGAAGC-3' (product size: 346 bp cDNA); *AgOr40*: f5'-GACCCTCAAGAACCAGGGCT-3' and r5'-AATGATGGTGTAGTACGAGAAGG-3'; *AgIr76b*: f5'-ATCTTCGATCCAGAGTTGCT-3' and r5'-CCGGTCACCATGACGAAGTA-3'. qRT-PCR was carried out using an Applied Biosystems 7300 Real-time PCR system and SYBR green as fluorescent dye. Three experimental repetitions were analyzed for each biological sample and the data processed using System 7300 Sequence Detection Software (version 1.3.1). Primer efficiency was determined using a standard curve for all the primers used. In the amplification of target genes and *rps7*, 8 μ l and 2 μ l cDNA, respectively, from each group were used as templates. In each

trial, cDNA levels of target genes were quantified relative to *rps7* levels using the method of Pfaffl²¹.

Results

Behavioral Responses of Individual Larva

Previous studies utilized a novel paradigm to assay the behavioral responses of large groups of *An. gambiae* late instar larvae to various natural and synthetic odorants in order to characterize the molecular and cellular elements of the larval olfactory system¹⁵. While providing fundamental information about the components underlying the olfactory responses of *An. gambiae* larvae, these end-point studies did not provide the precise tracking information that would allow us to distinguish between attractive or repulsive behavioral patterns. In addition, the need for a large number of larvae precluded its use in other experimental contexts. To provide such information and utility, a CCD camera-based tracking system was utilized to study the behavior of individual *An. gambiae* larva in response to odorant stimuli. Visual tracking records (Figure 2) were then analyzed to distinguish parameters associated with directional movement. These included calculating the total number of turns, the overall number of

movements, the average velocity, and the resting time for each larval behavioral assay (Figures 2 and 3).

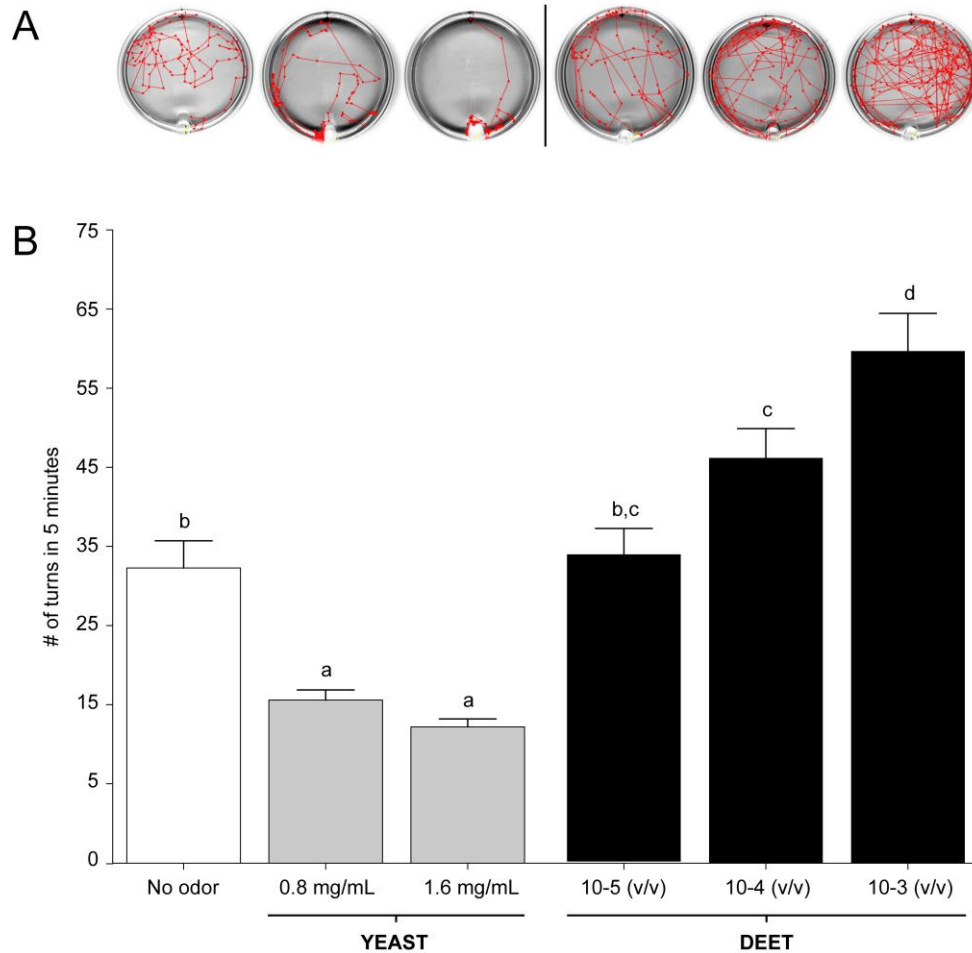


Figure 2. Larval responses in *An. gambiae* to yeast and DEET elicit opposite behaviors.

(A) 2-D tracking maps (top view) of freely moving individual larva during a 5 min time lapse. (B) Average number of turns exhibited by larvae in response to no odor, two concentrations of yeast paste, and three concentrations of DEET were assessed independently over a 5 min time lapse. Treatments with high DEET concentrations (10^{-4} and 10^{-3} v/v dilutions) and yeast paste (0.8 and 1.6 mg/ml) differed significantly from the no-odor control ($p < 0.01$). Results are shown as means \pm SE, $n = 10$.

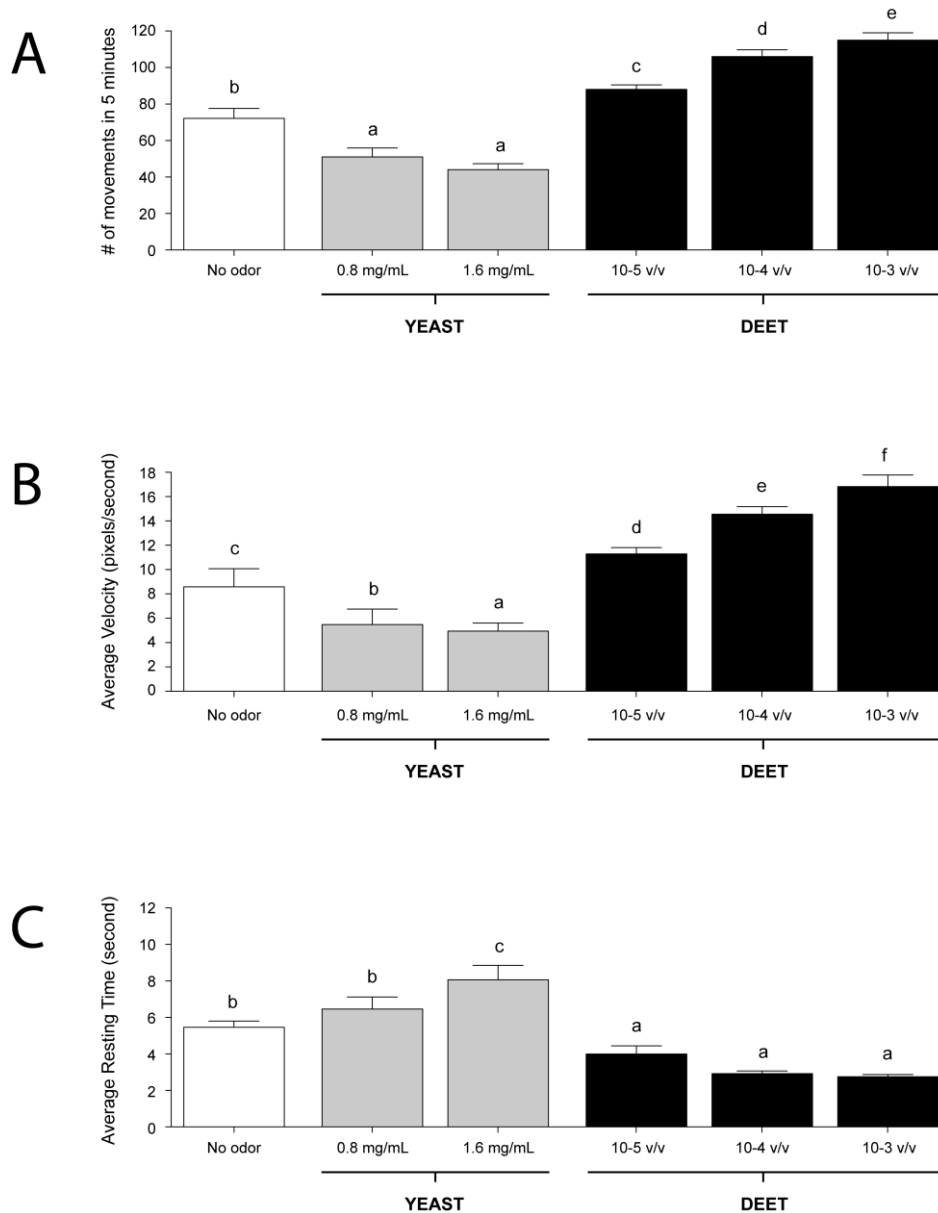


Figure 3. Behavioral effects of yeast and DEET on *An. gambiae*. Larval responses to yeast and DEET stimuli. Average number of movements (A), velocity (B), and resting time (C)—histograms of larval responses to two concentrations of yeast paste and three concentrations of DEET. Compared with the no-odor control, yeast, and DEET significantly affected larval activity ($p < 0.05$). Results are shown as means \pm SE, $n = 10$.

The sensitivity of this system was initially tested with two odorant stimuli, each of which evoked a strong dose-dependent response in the *An. gambiae* larvae group assay paradigm¹⁵. The first was DEET, which is a widely used commercial insect repellent. The second was yeast paste, a complex odorant source and a normal component of larval food. The behavioral responses of individual *An. gambiae* larva to three concentrations of DEET and two concentrations of yeast paste were examined along with the appropriate set of parallel no-odorant controls (Figure 2). For each assay, the four behavioral parameters described above were quantified. In these studies, yeast paste elicited decreases in overall larval turning (inverse klinokinesis; Figure 2) and movement (Figure 3) as well as concomitant increases in resting time when compared with no-odorant controls. In contrast, DEET elicited nearly the opposite effect: *An. gambiae* larvae displayed a dose-dependent increase in the turning rate (direct klinokinesis; Figure 2), number of movements, and average velocity (direct orthokinesis; Figure 3), while the average resting time was reduced to threshold levels at dilutions of 10^{-3} and 10^{-4} .

To confirm that the odorant-evoked behavioral responses were mediated by the larval olfactory system, a parallel set of assays were carried out after hand dissection of both larval antennae to effectively eliminate the site of olfactory signal transduction. Antennal-ablated larvae

appeared to be largely indifferent to high concentrations of both DEET and yeast, as larval responses were indistinguishable from no-odorant and unablated controls (Figure 4). In larvae in which the antennae were left intact but maxillary palps removed, responses to DEET and yeast paste were similar to those in unablated controls (Figure 4). Taken together, these data demonstrate that we have developed a robust behavioral paradigm for examining odorant-induced responses from individual *An. gambiae* larva.

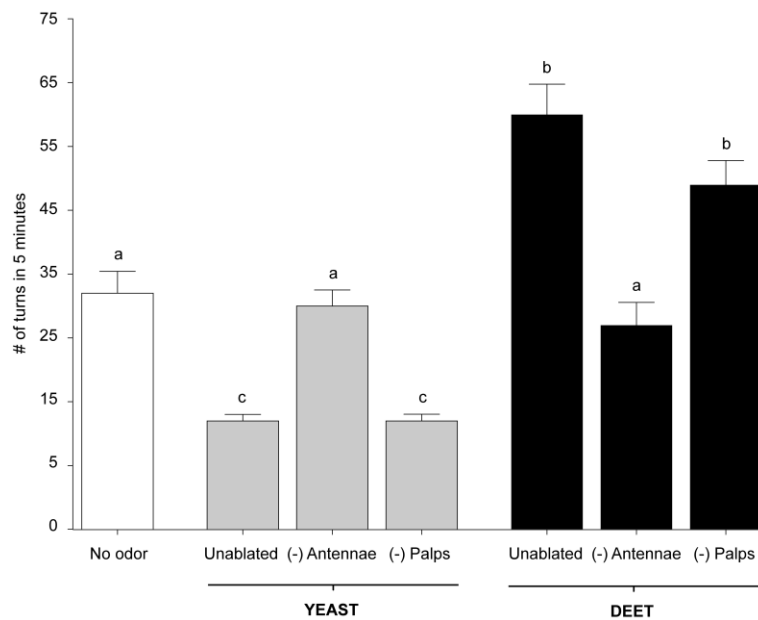


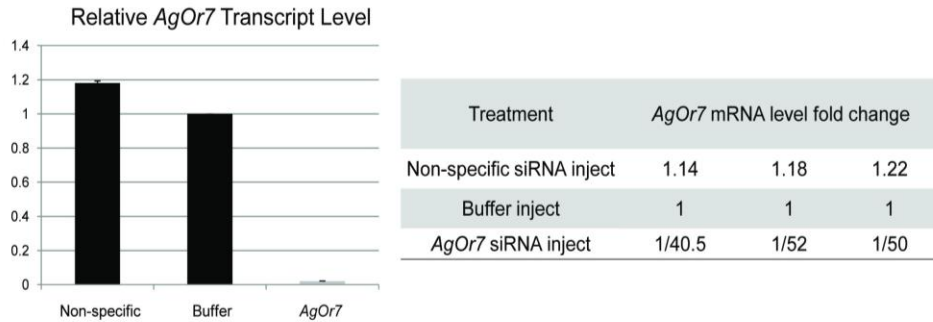
Figure 4. Larval antennae mediate responses to yeast and DEET. In the presence of yeast and DEET, unablated and palp-ablated larvae responded equally to both; ablation of the antennae, however, significantly increased or decreased the number of turns ($p < 0.05$) in response to yeast and DEET, respectively. Results are shown as means \pm SE, $n = 10$.

AgORs Silencing Confirms a Direct Role in the DEET Response

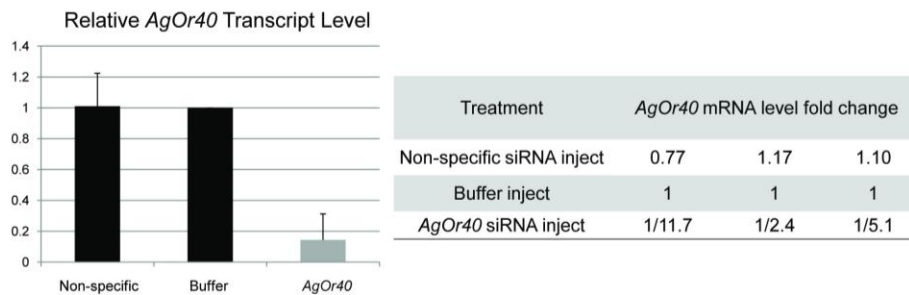
To discern the molecular basis for odorant-evoked behavioral responses of *An. gambiae* larvae, we initially focused on the role of *AgOrco*, which is the *An. gambiae* ortholog of the non-conventional *Drosophila* OR, *DmOrco*^{6,8}, and is highly expressed in the larval antenna¹⁵. In the absence of effective strategies to generate mutant or transgenic strains of *An. gambiae*, we used RNA interference (RNAi) to reduce *AgOrco* mRNA levels in individual larva, which could then be tested for abnormal behavioral responses. Individual larval behavioral assays followed by quantitative RNA analyses were conducted to assess the effects of *AgOrco* siRNA and control siRNA microinjections on olfactory responses and transcript levels. To account for non-specific effects of siRNA delivery, larvae were microinjected with identical amounts of a siRNA designed against a gene (AT5G39360) from the *Arabidopsis thaliana* genome lacking significant homology to any cDNA in *An. gambiae*. Furthermore, buffer-alone microinjections were carried out in parallel to assess any potential effects of microinjection on larval behavior.

In order to assess the efficiency of siRNA-mediated knockdown of *AgOrco* transcripts, a series of qRT-PCR studies were carried out on experimental and control larvae after behavioral testing. In these assays, cDNA was prepared from larval heads (with olfactory antennae attached)

from individual larva collected immediately following behavioral testing. These data (Figure 5) confirm that microinjection of siRNAs targeting *AgOrco* resulted in dramatic decreases in levels of this transcript.



Gene	Treatment	Cycle Threshold (CT) Value		
<i>Rps7</i> Primer Effi. = 2	Non-specific siRNA injected	22.28	22.35	22.16
	<i>AgOr7</i> siRNA injected	22.67	22.74	22.43
	Buffer injected	16.00	16.07	16.05
<i>AgOr7</i> Primer Effi. = 2	Non-specific siRNA injected	29.42	29.64	29.47
	<i>AgOr7</i> siRNA injected	35.34	36.00	35.70
	Buffer injected	23.33	23.60	23.65



Gene	Treatment	Cycle Threshold (CT) Value		
<i>Rps7</i> Primer Effi. = 2	Non-specific siRNA injected	17.10	17.21	17.25
	<i>AgOr40</i> siRNA injected	17.12	17.48	17.09
	Buffer injected	17.13	17.25	17.22
<i>AgOr40</i> Primer Effi. = 1.91	Non-specific siRNA injected	14.34	14.76	14.53
	<i>AgOr40</i> siRNA injected	17.37	16.65	17.28
	Buffer injected	14.33	15.04	14.64

Figure 5. Quantitative analysis demonstrates significant transcript level reduction of *AgOrco* and *AgOr40* after siRNA treatment.

Larval cDNAs for qRT-PCR were generated using equal amounts (2 µg for *AgOrco* and 4 µg for *AgOr40*) of RNA extracted from hand-dissected larval heads from each injection treatment group, and three technical replicates were performed for each experimental group. *AgOrco* and *AgOr40* mRNA levels were quantified as fold-changes relative to *rps7* using the method of Pfaffl. *AgOrco* and *AgOr40* levels are shown after normalization to buffer-alone controls in each of three experimental replicates. Histograms showing averaged *AgOrco* and *AgOr40* levels normalized to buffer-alone injection controls. Standard errors were ± 0.041 and ± 0.029 for non-specific and *AgOrco* siRNA injections; ± 0.127 and ± 0.392 for non-specific and *AgOr40* siRNA injections, respectively. Raw data from each qRT-PCR reaction indicating cycle-threshold (CT) and primer efficiency information for each technical replicate.

Although a modest microinjection effect was observed on the average larval velocity, the overall number of turns (Figure 6) as well as the number of movements, average velocity, and resting time (Figure 7) in response to 1.6 mg/ml yeast paste stimuli were largely unaffected by microinjection with *AgOrco* or control siRNAs. In contrast, a 1×10^{-3} (v/v) dilution of DEET in individuals that received *AgOrco* siRNA showed significant ($p < 0.01$) reductions in turns (Figure 6), movements, and velocity as well as a significant increase in their average resting time relative to buffer-injected and control larvae (Figure 7). Although a modest microinjection effect was again observed in buffer-injected larvae, these results are consistent with the hypothesis that larval responses to DEET

are *AgOrco*-dependent whilst larval responses to yeast paste are *AgOrco*-independent.

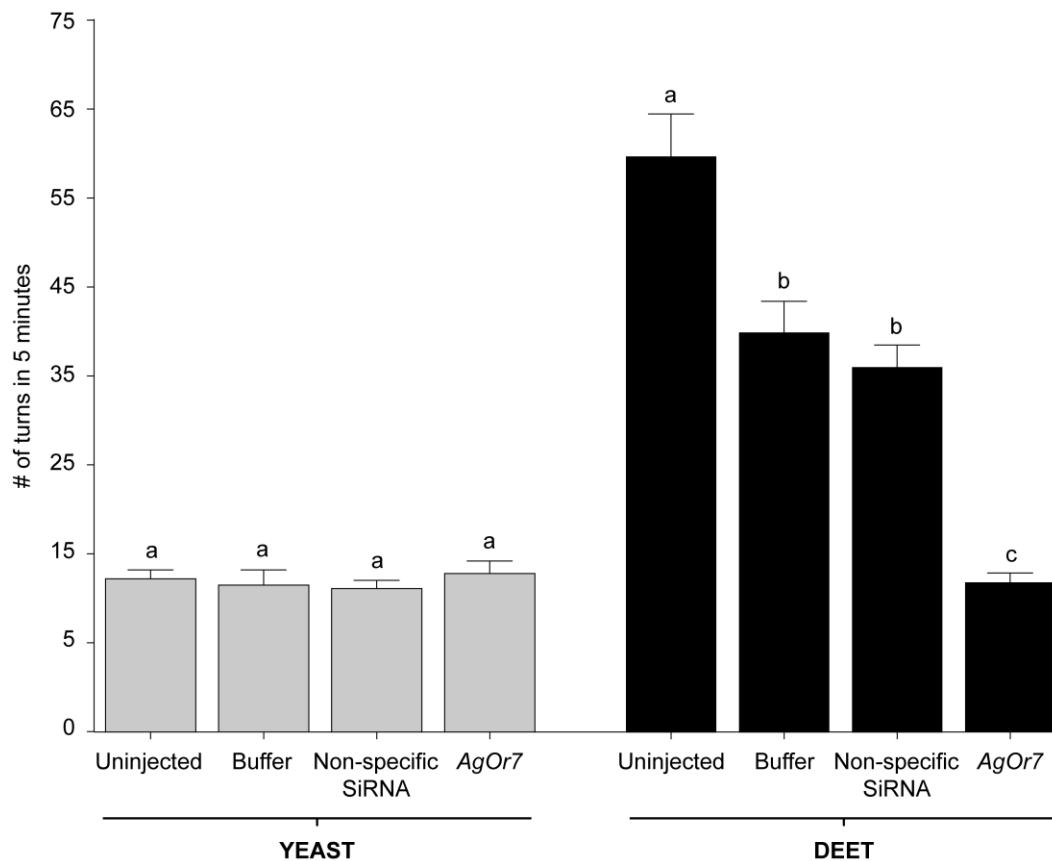


Figure 6. Differential sensitivity of larval responses in *An. gambiae* to siRNA-mediated knockdown of *AgOrco* is odorant dependent.

The average number of turns exhibited by uninjected larvae as well as those receiving mock (buffer-alone), non-specific, or siRNA injections in response to yeast paste and DEET were assessed independently over a 5 min time lapse. Larval responses to 1.6 mg/ml yeast paste were unaffected by any siRNA treatments (A) while larvae receiving *AgOrco* siRNAs displayed significant reductions in turning rates in response to a 10^{-3} v/v dilution of DEET (B). Buffer and non-specific siRNA-injected animals displayed a comparable reduction of the number of turns ($p < 0.05$). Results are shown as means \pm SE, $n = 10$.

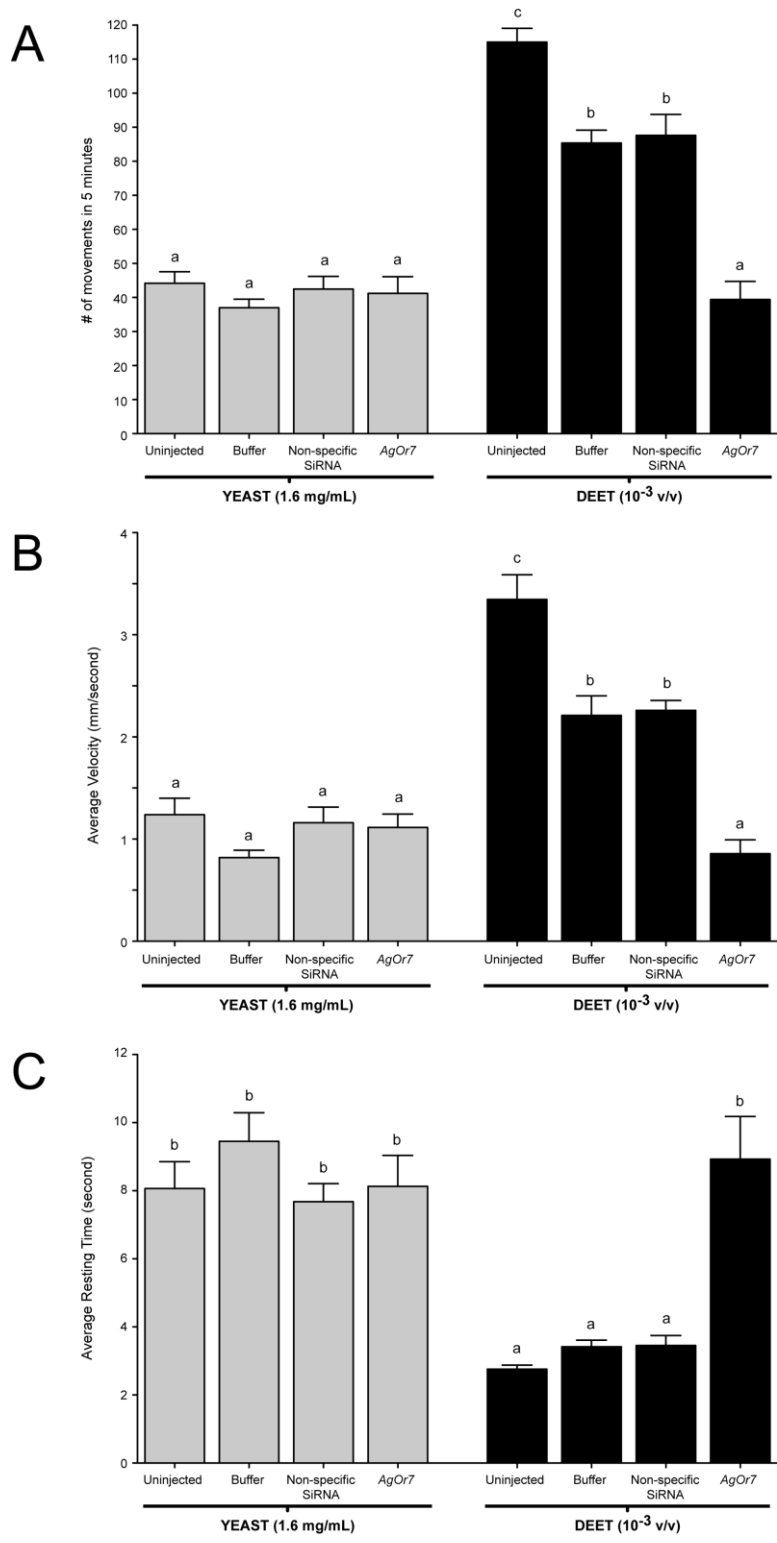


Figure 7. Larval behaviors after injection of non-specific small interfering RNA (siRNA).

Averaged responses of buffer, non-specific, and *AgOrco* siRNA-injected larvae in the presence of 1.6 mg/ml yeast paste and a 10^{-3} v/v dilution of DEET. Larval movement (A), velocity (B), and resting time (C) behaviors of larvae in response to yeast paste and DEET. Knockdown of *AgOrco* mRNA levels has no effect on the ability of larvae to respond to yeast paste yet evokes significant behavioral alterations in larval responses to DEET ($p < 0.01$). Results are shown as means \pm SE, $n = 10$.

Functional studies using *Xenopus* oocytes¹⁵ have previously identified AgOR40 as a conventional ligand-specific larval AgOR that responds to DEET stimulation and, by implication, is likely to be responsible for DEET-elicited behavioral responses in *An. gambiae* larvae. Inasmuch as the molecular basis for DEET mediated behaviors remains controversial, we tested this hypothesis by using siRNA-mediated gene silencing to examine whether knockdown of *AgOr40* transcripts would also perturb behavioral responses to DEET and yeast paste. In these studies, injection of siRNAs targeting *AgOr40* echoed the effects of *AgOrco* siRNAs and showed a significant reduction in turns and other elements of larval behavior in response to DEET stimuli (Figure 8A) and were unaffected in response to yeast paste (Figure 8B). As was the case for *AgOrco* silencing, qRT-PCR studies were carried out on experimental and control larvae after behavioral testing to assess the levels of *AgOr40* transcripts. These data (Figure 5) confirm that microinjection of siRNAs

targeting *AgOr40* resulted in dramatic decreases in *AgOr40* transcript levels without significantly altering *AgOrco* mRNA pools. Taken together, these data directly validate the role of AgOR40 as a DEET-specific conventional AgOR in the larval olfactory system of *An. gambiae*.

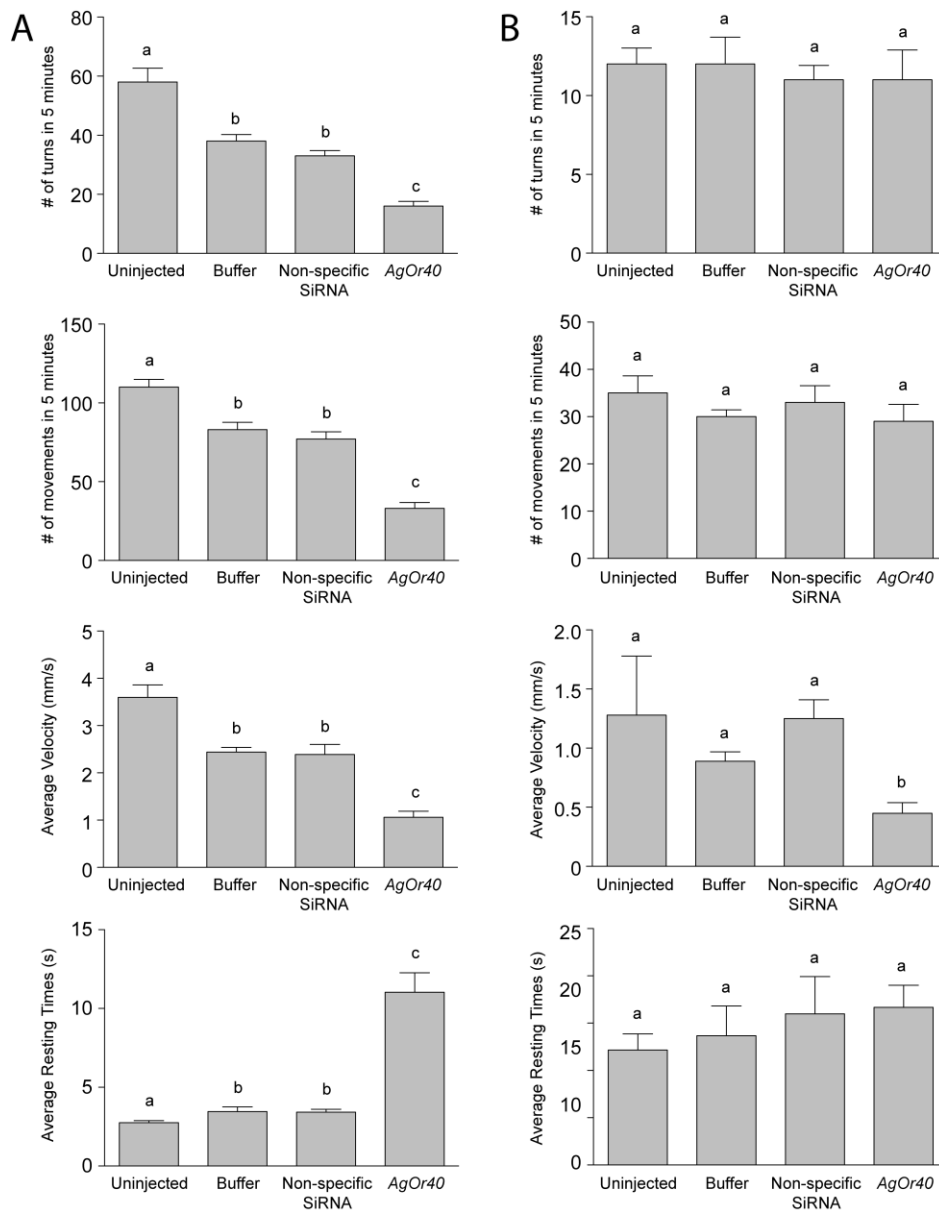


Figure 8. Differential sensitivity of larval responses in *An. gambiae* to siRNA-mediated knockdown of *AgOr40* is odorant dependent.

Larval responses exhibited by uninjected larvae as well as those receiving mock (buffer-alone), non-specific, or siRNA injections in response to DEET (A) and yeast paste (B) were assessed independently over a 5 min time lapse. Larval responses to 1.6 mg/ml yeast paste were unaffected by any siRNA treatments while larvae receiving *AgOr40* siRNAs displayed significant reductions in turning rates (top panel) in response to a 10^{-3} v/v dilution of DEET. Buffer and non-specific siRNA-injected animals displayed a comparable reduction of the number of turns ($p < 0.05$). Larval movement, velocity, and resting time behaviors (from top to bottom) of larvae in response to DEET (A) and yeast paste (B) where knockdown of *AgOr40* mRNA levels had no effect on the ability of larvae to respond to yeast paste yet evoked significant behavioral alterations in larval responses to DEET ($p < 0.01$). Results are shown as means \pm SE, $n = 10$.

AgIRs Mediate AgOR Independent Olfactory Responses

Based on the *AgOrco*-independent response of larvae to yeast paste, we next investigated whether *AgOrco*-dependent and -independent olfactory signaling exists in *An. gambiae* larvae. In doing so, we considered that *AgOrco* independence of the larval yeast response might, in part, reflect that yeast paste is a complex mixture, some components of which may activate *AgOrco*-independent olfactory signaling pathways. In contrast, DEET is a unitary compound that specifically elicits *AgOr*-dependent behavioral responses in *An. gambiae* larvae and physiological responses in *Xenopus* oocyte-based *AgOR* functional assays¹⁵. To examine further the possibility that distinct signaling pathways are active in this system, we searched the *An. gambiae* genome for homologs of

variant ionotropic glutamate receptors that have recently been shown to function as novel chemosensory proteins in *D. melanogaster* (DmIRs)¹³. We have identified a family of 46 *An. gambiae* variant ionotropic glutamate receptors, which we have named *AgamGLUVIRs*, and 9 homologs of ionotropic glutamate receptors, named *AgamGLURs* or *AgamNMDARs*, all according to the convention established by the *An. gambiae* genome consortium (www.Vectorbase.org). For convenience we refer to the *AgamGLUVIR* genes as *AgIrs* and their conceptual peptide products as AgIRs. Another group of researchers has independently identified the same family of genes²² and we have agreed with them on a unified nomenclature in order to avoid confusion in future publications. A listing of the entire gene family, their chromosome positions, and peptide sequences is given in Table 1.

Gene	peptide name	VectorBase ID
<i>AgamGLURI</i>	AgGLURI	AGAP006027
<i>AgamGLURIIa</i>	AgGLURIIa	AGAP000803
<i>AgamGLURIIb</i>	AgGLURIIb	AGAP000801
<i>AgamGLURIIc</i>	AgGLURIIc	AGAP000798
<i>AgamGLURIID</i>	AgGLURIID	AGAP002797
<i>AgamGLURIIe</i>	AgGLURIIe	AGAP012447
<i>AgamNMDAR1</i>	AgNMDAR1	AGAP001478
<i>AgamNMDAR2</i>	AgNMDAR2	AGAP012429
<i>AgamNMDAR3</i>	AgNMDAR3	AGAP005527
<i>AgamGLUvir7h.1</i>	AgIR7h.1	AGAP013154
<i>AgamGLUvir7i</i>	AgIR7i	AGAP013363
<i>AgamGLUvir7n</i>	AgIR7n	AGAP000714
<i>AgamGLUvir7s</i>	AgIR7s	AGAP013409
<i>AgamGLUvir7t</i>	AgIR7t	AGAP002763
<i>AgamGLUvir7u</i>	AgIR7u	AGAP013285
<i>AgamGLUvir7w</i>	AgIR7w	AGAP013416
<i>AgamGLUvir7x</i>	AgIR7x	AGAP013520
<i>AgamGLUvir7y</i>	AgIR7y	AGAP013172
<i>AgamGLUvir8a</i>	AgIR8a	AGAP010411
<i>AgamGLUvir21a</i>	AgIR21a	AGAP008511
<i>AgamGLUvir25a</i>	AgIR25a	AGAP010272
<i>AgamGLUvir31a</i>	AgIR31a	AGAP009014
<i>AgamGLUvir40a</i>	AgIR40a	AGAP004021
<i>AgamGLUvir41a</i>	AgIR41a	AGAP002904
<i>AgamGLUvir41b</i>	AgIR41b	AGAP008759
<i>AgamGLUvir41c</i>	AgIR41c	AGAP012951
<i>AgamGLUvir41n</i>	AgIR41n	AGAP003531
<i>AgamGLUvir41t.1</i>	AgIR41t.1	AGAP004432
<i>AgamGLUvir41t.2</i>	AgIR41t.2	AGAP012969
<i>AgamGLUvir60a</i>	AgIR60a	AGAP011943
<i>AgamGLUvir64a</i>	AgIR64a	AGAP004923
<i>AgamGLUvir68a</i>	AgIR68a	AGAP007951
<i>AgamGLUvir75d</i>	AgIR75d	AGAP004969
<i>AgamGLUvir75g</i>	AgIR75g	AGAP013085
<i>AgamGLUvir75h.1</i>	AgIR75h.1	AGAP001811
<i>AgamGLUvir75h.2</i>	AgIR75h.2	AGAP001812
<i>AgamGLUvir75k</i>	AgIR75k	AGAP007498
<i>AgamGLUvir75l</i>	AgIR75l	AGAP005466
<i>AgamGLUvir76b</i>	AgIR76b	AGAP011968
<i>AgamGLUvir93a</i>	AgIR93a	AGAP000256
<i>AgamGLUvir100a</i>	AgIR100a	AGAP000140
<i>AgamGLUvir100h</i>	AgIR100h	AGAP000293

<i>AgamGLUvir100i</i>	AgIR100i	AGAP004475
<i>AgamGLUvir101</i>	AgIR101	AGAP013425
<i>AgamGLUvir133</i>	AgIR133	AGAP005677
<i>AgamGLUvir134</i>	AgIR134	AGAP005678
<i>AgamGLUvir135</i>	AgIR135	AGAP005679
<i>AgamGLUvir136</i>	AgIR136	AGAP006440
<i>AgamGLUvir137</i>	AgIR137	-
<i>AgamGLUvir138</i>	AgIR138	-
<i>AgamGLUvir139</i>	AgIR139	AGAP006691
<i>AgamGLUvir140.1</i>	AgIR140.1	AGAP013242
<i>AgamGLUvir140.2</i>	AgIR140.2	AGAP013436
<i>AgamGLUvir141</i>	AgIR141	AGAP013473
<i>AgamGLUvir142</i>	AgIR142	AGAP006407

Table 1. Annotation of AgIR family members.

Nomenclature, chromosome positions, and conceptual peptide sequences of ionotropic glutamate (AgamGLUR and AgamNMDAR) and variant ionotropic glutamate receptor (AgamGLUvir) families in *An. gambiae*. Column headers indicate: (1) long form of gene name; (2) short form of peptide name; (3) VectorBase gene identification number; (4) chromosome location and base pair position (plus, + or minus, – strand in parentheses) of updated gene annotation; and (5) conceptual peptide sequence of new gene model (single letter amino acid code). AgGLUR1 and AgGLUR1lb represent partial peptides where the 5' end of the gene has not been annotated.

A phylogenetic reconstruction comparing the amino acid sequences of AgIRs and DmIRs shows deep branching and low bootstrap support for many of the implied relationships, reflecting the considerable sequence diversity between these proteins both within and across species (Figure 9). The most convincing relationships are observed within the iGluRs, suggesting conservation of function (Figure 9). Very few strong homologs are observed between AgIRs and DmIRs. Despite their

diversity, topology predictions indicate conservation of 4 hydrophobic stretches of amino acids that likely correlate to the transmembrane and pore regions (Figure 10) of known ionotropic glutamate receptors.

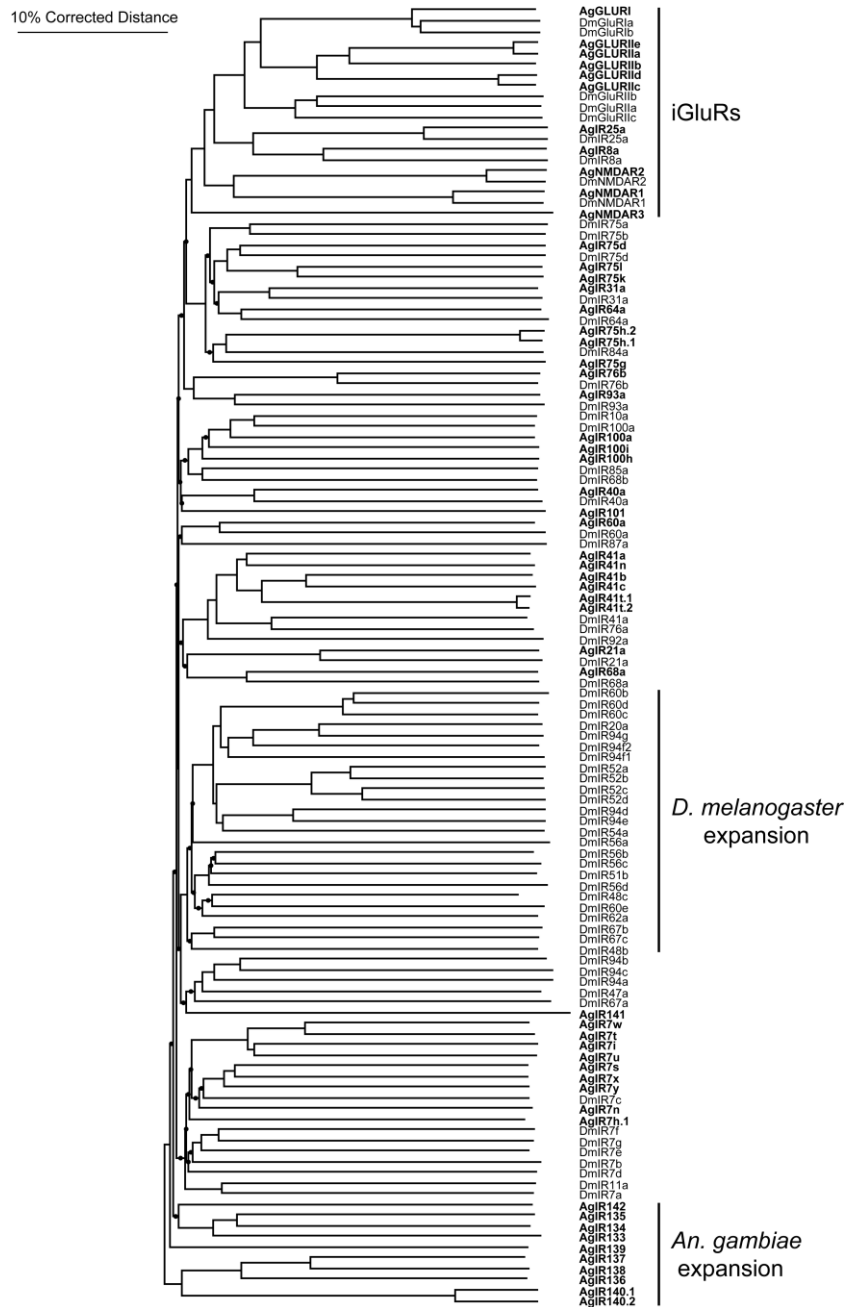


Figure 9. AgIR/DmiR phylogenetic tree. Neighbor-joining tree based on amino acid alignments of AgIR and DmiR peptides. AgIR names are shown in bold type and DmiR names are shown in plain type. Black dots indicate branch points where bootstrap support is less than 50%.

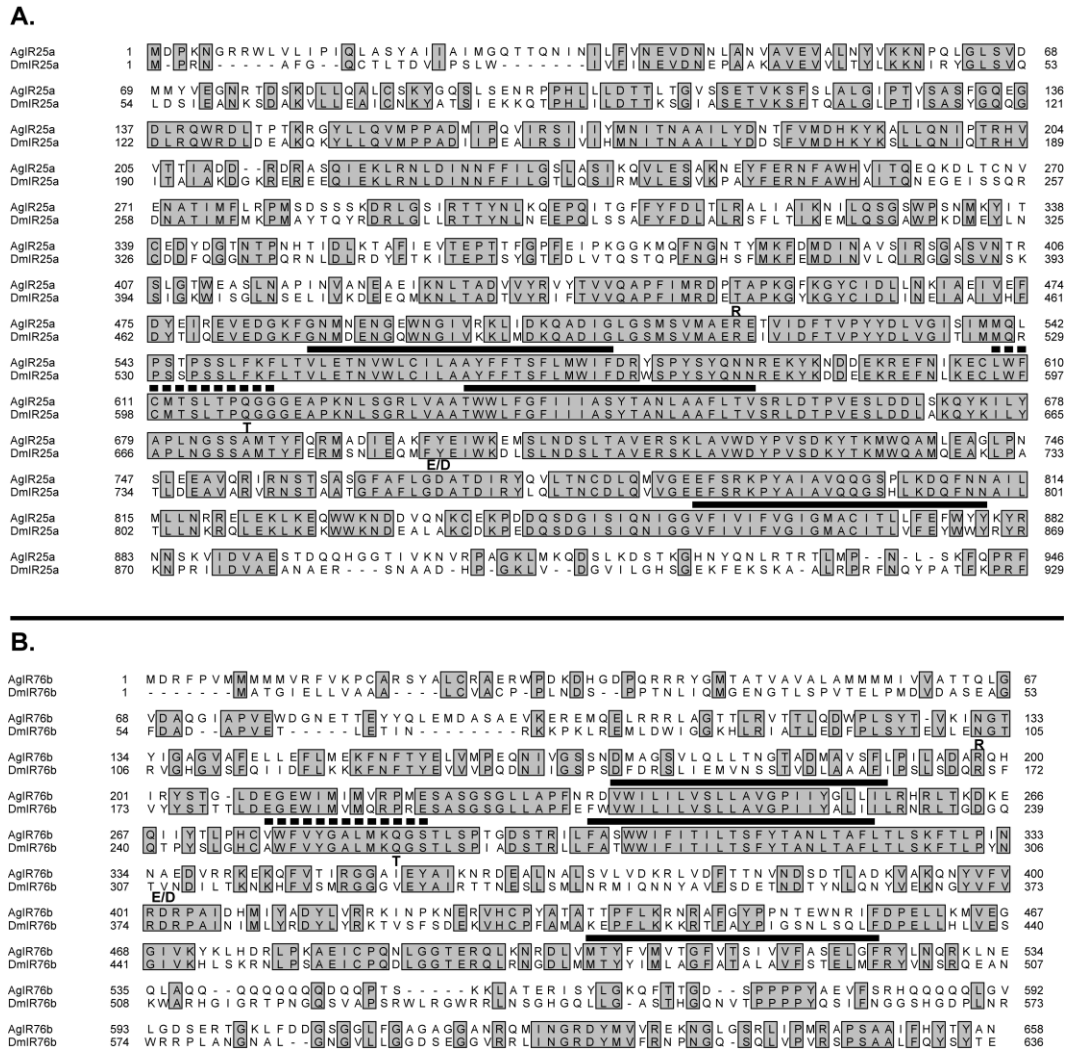


Figure 10. Representative alignments of AgIR and DmIR homologs. (A) IR25a peptide alignment. (B) IR76b peptide alignment. Amino acid sequences (single letter code) were aligned using ClustalX. Identical residues are shaded. Bold lines above residues indicate predicted transmembrane helices, while the dotted line above residues indicates the potential pore loop. Boldface letters represent amino acids arginine (R), threonine (T), or glutamic acid/aspartic acid (E/D) at positions that are found in known glutamate receptors.

Interestingly, two of the strongest AgIR homologs of DmIRs are found within the iGluR clade (Figure 8). AgIR25a shares 68% amino acid identity (84% similarity) with DmIR25a, and AgIR8a shares 42% identity (63% similarity) with DmIR8a, genes that are broadly expressed in coeloconic sensilla neurons in the third antennal segment of *D. melanogaster*. These 2 peptides are also much longer, 891aa and 946aa, respectively, than other AgIRs (average length 664aa) and are closer in size to the iGluRs (avg. 974aa, including partial peptides). Moreover, AgIR25 has retained 2 of the 3 amino acids, an arginine and an aspartic acid (Figure 10A), in positions that are known to be important for glutamate binding. Importantly, some classes of NMDA receptors also lack the 3rd residue²³. AgIR8a has potential glutamate-binding residues in all three conserved positions, while several other AgIRs, including AgIR76b, retain one or more (Figure 10B). Most other AgIRs are divergent at those positions.

As a first step toward characterizing the potential role of AgIRs in larval olfactory signaling, we carried out RT-PCR using cDNA derived from *An. gambiae* larval antennae and gene-specific primers to 5 Aglr genes. These studies indicated that multiple members of this class of candidate chemosensory genes are expressed in the larval antenna (Figure 11) as 4 of the 5 Aglrs could be amplified from larval antennae. Additionally, expression of one member of the ionotropic glutamate receptor family, AgNMDAR2, was observed in larval antennae (Figure 11). We expect future work to elucidate the expression profiles of all Aglrs in both the larval and adult olfactory tissues of *An. gambiae*.

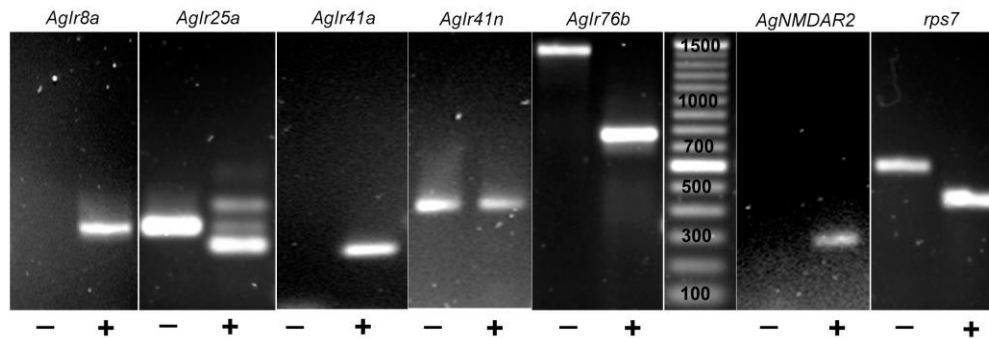


Figure 11. Expression of Aglrs in larval antennae.

Composite image of agarose gel lanes showing cDNA (lower) and gDNA (upper) bands following RT-PCR using Aglr-specific primers as indicated above lanes. Minus (-) and plus (+) signs below lanes indicate the presence or absence of reverse transcriptase in first strand cDNA synthesis reaction, respectively. Bands (base pairs): Aglr8a cDNA (319); Aglr25a cDNA (271), gDNA (334); Aglr41a cDNA (245); Aglr41n cDNA (336, not present), gDNA (417); AgNMDAR2 cDNA (328); Aglr76b cDNA (770), gDNA (1414); rps7 cDNA (460), gDNA (609). No genomic bands were expected for Aglr8a, Aglr41a, and AgNMDAR2 as the forward primers spanned an exon-exon junction. All bands that appeared in gels are shown and Photoshop was used only to adjust the brightness and contrast of each panel. Marker lane shows 100 bp ladder (New England Biolabs).

In order to examine whether AgORs and AgIRs perform distinct functional roles in the olfactory system of *An. gambiae*, we carried out behavioral assays using two additional unitary odorants that have been used successfully in previous behavioral and functional studies¹⁵. The first was 3-methylphenol (3MP), which was shown to activate AgOR-dependent pathways and evoke robust behavioral responses in larvae. In our current studies, larvae manifest dose-dependent reductions in turns and overall movement, as well as threshold-dependent increases in

average resting time (Figure 12). Furthermore, larval responses to 10^{-4} dilutions of 3MP were significantly altered in larvae injected with *AgOrco* siRNA, whereas control or buffer-injected larval responses were statistically equivalent to uninjected control larvae (Figures 13 and 14A). AgOR40 is one of 3 larval AgORs with a demonstrated sensitivity to 3MP. In that light, we also tested the ability of siRNA mediated silencing of *AgOr40* expression to alter larval responses to 3MP—in these studies a marginal but not statistically significant effect was observed (unpublished data) that is consistent with the role of multiple AgORs in mediating larval sensitivity to 3MP.

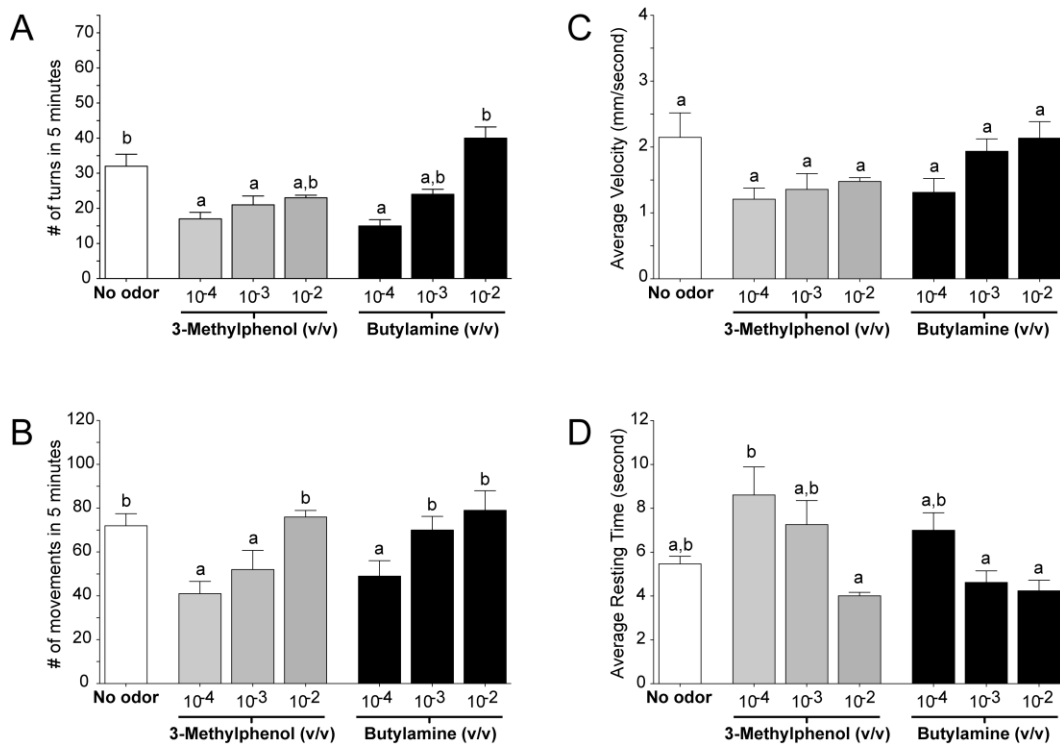


Figure 12. Behavioral effects of 3MP and Butylamine on *An. gambiae*. Larval responses to increasing dilutions (v/v) of 3MP and butylamine are displayed: total number of turns/assay (A), average number of movements/assay (B), average velocity (C), and resting time (D). With the exception of average velocity, for which no significant effects were detected, both odorants evoked dose-dependent responses on larval activity when compared with the no-odor control ($p < 0.05$). Results are shown as means \pm SE, $n = 10$.

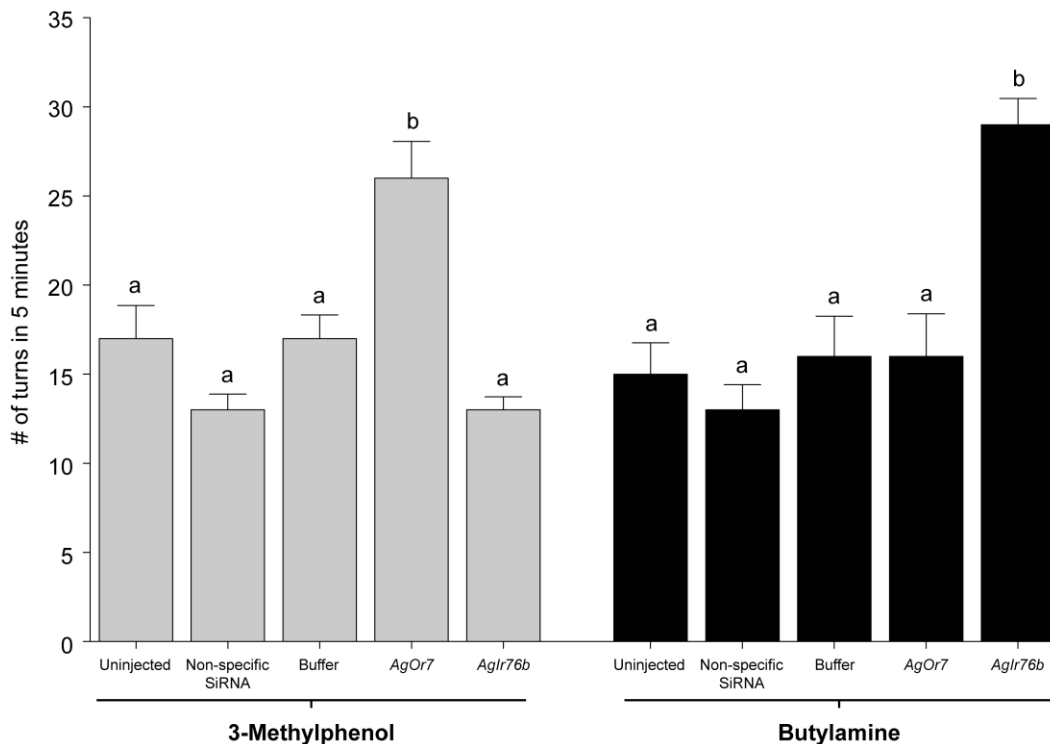
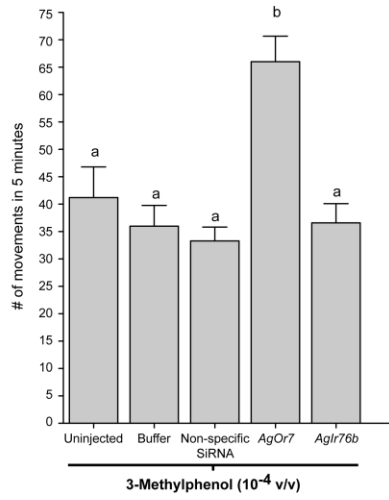


Figure 13. Olfactory responses to 3-methylphenol and butylamine are mediated by distinct signaling pathways. The turning rates exhibited by uninjected larvae as well as those receiving mock (buffer-alone), non-specific, or siRNA injections in response to 10^{-4} v/v dilutions of 3-methylphenol or butylamine were assessed independently over a 5 min time lapse. (A) Larval responses to 3-methylphenol were significantly altered by *AgOrco* knockdown but unaffected by *AgIIR* silencing. (B) Conversely, responses to butylamine were sensitive to reduction in *AgIIR76b* mRNA levels but indifferent to silencing of *AgOrco* expression.

The next set of studies employed butylamine, a unitary odorant which has been shown to activate grooved-peg ORNs in *An. gambiae*²⁴ and *Culex quinquefasciatus* mosquitoes²⁵. As was the case for 3MP, uninjected *An. gambiae* larvae displayed robust dose-dependent responses to butylamine (Figure 12). In contrast to the *AgOrco*-dependent nature of larval responses to 3MP, larval responses to butylamine were indistinguishable among animals treated with *AgOrco* and control siRNAs or microinjected with buffer alone (Figures 13 and 14B).

Based on their homology to DmIRs, which have been shown to mediate responses to amines and other odorants in *Drosophila*¹³, we postulated that AgIRs mediate larval responses to butylamine. To test this hypothesis, siRNA-mediated gene knockdowns were used in an attempt to silence larval AgIRs and subsequently examine the responses of larvae to butylamine. Of the *AgIrs* tested, microinjection of only one *AgIrr76b* siRNA displayed siRNA-specific effects on larval responses to butylamine. Microinjection of *AgIrr76b* siRNAs reduced *AgIrr76b* mRNA levels (Figure 15) and led to significant alterations in larval responses to butylamine (Figures 13 and 14B). Larval responses to butylamine were unaffected in *AgOrco* knockdowns and by microinjection of non-specific siRNAs or buffer-alone controls (Figures 13 and 14B).

A



B

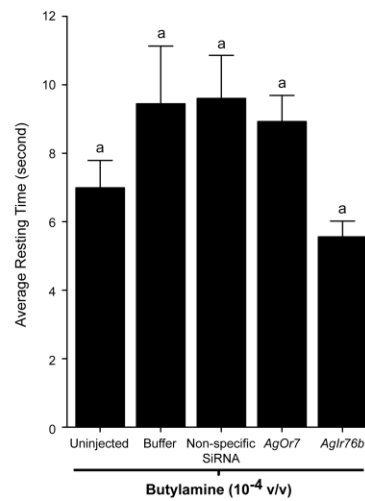
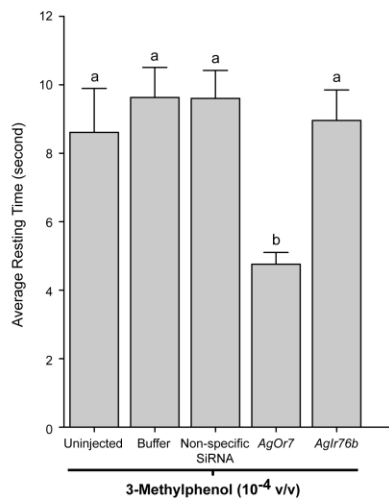
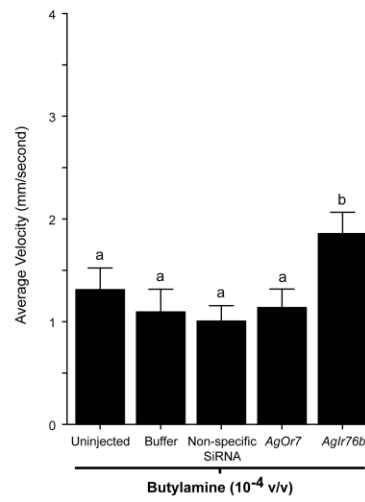
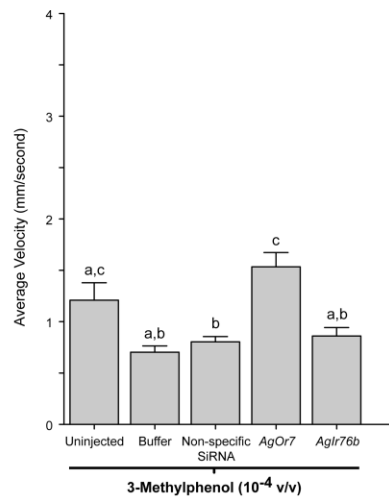
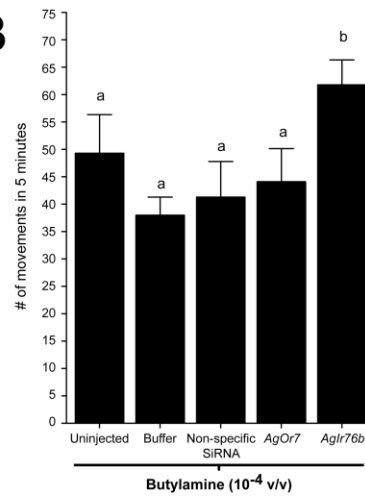


Figure 14. Odorant-specific differential effects of *AgOr/AgIr* knockdown. Averaged responses of buffer, non-specific, *AgOrco*, and *AgIr76b*-siRNA injected larvae in the presence of 10^{-4} v/v dilutions of 3-methylphenol (3MP, left panels) or butylamine (BA, right panels). Histograms of larval movement (A), velocity (B), and resting time (C) are presented. Knockdown *AgOr7* mRNA in larvae displayed significant behavioral alterations in response to 3MP without affecting BA-evoked behavior. Conversely, reduction of *AgIr76b* levels altered larval responses to BA without significantly affecting 3MP responses. Alteration of behavioral responses did not occur in the controls ($p < 0.05$). Results are shown as means \pm SE, n = 10.

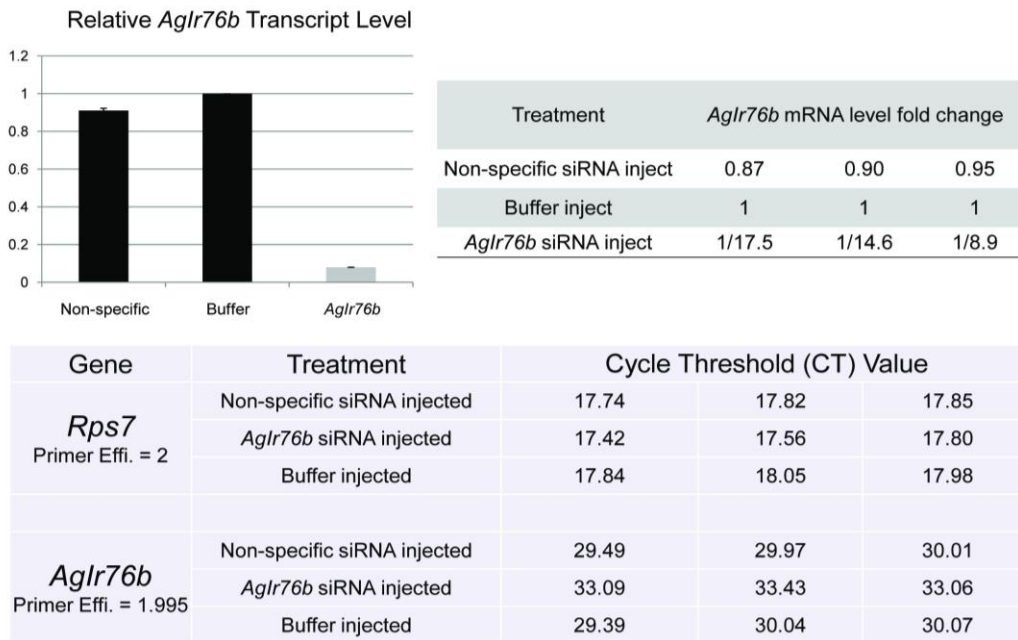


Figure 15. Quantitative mRNA analysis demonstrates significant transcript level reduction of *AgIr76b* after siRNA treatment. Larval cDNAs for qRT-PCR were generated using equal amounts (~3.5 μ g) of RNA extracted from hand-dissected larval heads from each injection treatment group. Two independent biological replicates were performed, each consisting of three technical replicates for every experimental group. *AgIr76b* mRNA levels were quantified as fold-changes relative to *Rps7* using the method of Pfaffl. *AgIr76b* levels are shown as averaged values of both biological replicates after normalization

to buffer alone controls in each of three technical replicates. Histograms showing averaged *AgIr76b* levels normalized to buffer alone injection controls. Standard errors were ± 0.04 and ± 0.003 for non-specific and *AgIr76b* siRNA injections, respectively. Raw data from each qRT-PCR reaction indicating cycle-threshold (CT) and primer efficiency information for each biological/technical replicate.

Discussion

In the face of a dearth of traditional genetic tools and a robust transgenic capacity, the ability to carry out RNAi-mediated gene silencing on individual *An. gambiae* larva provides an opportunity to examine the molecular basis for olfactory driven behaviors in this disease vector. Furthermore, the relative simplicity of the larval nervous system provides a considerably more tractable model within a non-model system for understanding similar processes that are presumed to underlie chemosensory responses in adults that directly contribute to Anopheline vectorial capacity.

In this study, we have developed a simple behavioral paradigm that can be used to track the olfactory responses of individual *An. gambiae* larva to a range of chemical stimuli. Overall, these data are consistent with the hypothesis that when larvae are exposed to a repellent compound, such as DEET, they exhibit an increased rate of turning and a rise in overall movement and velocity. In contrast, an attractant such as yeast

paste or 3MP leads to a reduction in the number of movements, turns, and average velocity while the average resting time is increased.

Together with gene-silencing approaches, we have employed a novel behavioral assay to provide compelling *in vivo* evidence that, for the first time, supports a direct *in vivo* role of AgORs in olfactory processes in *An. gambiae*. Furthermore, these studies go further to address the molecular mechanism responsible for DEET mediated repulsion of insects. Previous studies²⁶ suggesting that DEET's mode of action is to inhibit the activation of a subset of insect ORs that would otherwise be activated by attractants are in contrast to models that suggest DEET acts via direct excitation of OR-expressing ORNs that, in turn, evoke downstream behavioral repulsion. The excito-repellent hypothesis is consistent with our previous study on the larval olfactory system in *An. gambiae* that showed robust DEET-mediated behavioral responses that correlated with a discrete population of larval ORNs co-expressing AgORco/AgOR40 as well as specific DEET stimulation of *Xenopus* oocytes injected with AgORco/AgOR40 cRNAs. This hypothesis is also supported by other studies that describe DEET-mediated activation of a subset of ORNs in *Culex* mosquitoes²⁷ and more recent work in *Aedes aegypti* suggesting that DEET sensitivity is a genetically determined characteristic affecting the functionality of discrete ORNs²⁸. While the

reduction in DEET-mediated repellent responses in larvae undergoing RNAi mediated silencing of *AgOrco* is consistent with a general requirement for AgOR-based signaling, the similar effects of *AgOr40* silencing specifically supports the role of both these molecular targets in mediating DEET repellency. That these behavioral effects were manifest by DEET alone, *i.e.* in the absence of any other stimuli, further validates our earlier study and supports a direct excito-repellent mechanism for DEET activity.

Lastly, these studies uncover the existence of at least two parallel chemosensory transduction systems in larval-stage *An. gambiae* that respond to distinct classes of odorant stimuli. One pathway, which is in keeping with the established literature for insect olfactory signal transduction, is based on the obligatory role of the non-conventional Anopheline Orco family member *AgOrco*, which acts together with other conventional AgORs in the formation of functional receptors. It is likely that AgOR-dependent signaling pathways impact responses to a wide range of odorant cues that play important roles in several aspects of Anopheline behavior. These pathways are exemplified by the dramatic alterations in the DEET and 3MP responses of *An. gambiae* larvae after RNAi-mediated silencing of *AgOrco* transcripts (Figures 5, 13). The other pathway depends on the function of the *AgIr* gene family, which likely recognizes

different odor classes than the *AgOr* pathway. Moreover, the similarities between AgIRs 8a and 25a and iGluRs suggest that cellular receptors for glutamate in the antenna could act as a neuromodulator of ORN function. This hypothesis is consistent with the inability of *AgIr25a* siRNAs to alter larval behavioral responses to odors (unpublished data).

Recent functional analyses^{18,19} of AgOR-based odor coding against a diverse panel of compounds suggest that, in *An. gambiae*, olfactory pathways respond to a wide range of odorant stimuli with particular affinity for heterocyclics and aromatics that are associated with human skin emanations^{16,17}. These groups of odorants are thought to play essential roles in host-seeking, oviposition, and other behaviors that are critical for Anopheline life cycles²⁹. Coincidentally, this AgOR-based odor space is characterized by sparse responses to the majority of acids, aldehydes, and esters that were tested in addition to being particularly devoid of amine-elicited responses. This raised the suggestion that sensitivity to these classes of odorants might lie outside of *AgOr*-dependent olfactory signaling pathways.

We have identified several *AgIrs* that are expressed in larval olfactory tissues (Figure 11) and have used RNAi-mediated gene silencing to demonstrate the role of one of these genes in mediating larval responses to the AgOR-independent odorant butylamine. Critically, while

knockdown of *AgIr76b* specifically altered larval responses to butylamine, there was no effect on responses to two other unitary odorants that were dependent on *AgOrco* expression. These data are consistent with the hypothesis that, in contrast to the AgOR-dependent sensitivity to 3MP, DEET, and a broad range of “general” odorants, Anopheline responses to other odorants (e.g., butylamine) are mediated through *AgIr*-dependent signaling. There is reason to assume that these parallel pathways persist through to adult *An. gambiae* where *AgIrs* are likely to be responsible for olfactory sensitivity to important human kairomones, such as ammonia and lactic acid that are known to activate ORNs in grooved peg sensilla³⁰ that are devoid of *AgOrco*. Indeed, we have observed expression of multiple *AgIrs* in adult olfactory appendages, supporting the hypothesis that this family of genes is involved in chemosensory signaling in adults (manuscript in preparation).

Current efforts are directed toward expanding our understanding of *AgIr*-based odor coding in *An. gambiae*. Improving our understanding of olfactory signal transduction in *An. gambiae* may lead to new opportunities to target olfactory mediated behaviors at the molecular level. In turn, this may reduce the vectorial capacity of *An. gambiae* and help reduce the transmission of malaria and other important human diseases.

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Author Contributions

The author(s) have made the following declarations about their contributions: Conceived and designed the experiments: CL RJP LJZ. Performed the experiments: CL RJP. Analyzed the data: CL RJP LJZ. Contributed reagents/materials/analysis tools: RJP JDB PLJ GW. Wrote the paper: CL RJP JDB PLJ LJZ.

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CHAPTER III

MOLECULAR CHARACTERIZATION OF LARVAL PERIPHERAL THERMOSENSORY RESPONSES OF THE MALARIA VECTOR MOSQUITO *ANOPHELES GAMBIAE*

Preface

The following article was published was published in the journal of PLoS One (8(8): e72595. doi:10.1371/journal.pone.0072595) on which I was the first author along with Laurence J. Zwiebel (corresponding author). In this paper I have developed a thermo-electric apparatus to investigate thermosensory-driven behavior in *An. gambiae* larvae and utilized Whole-mount Fluorescent In-situ Hybridization (WM-FISH) as well as RNA interference to probe the localization and function of a thermosensory channel protein: TRPA1, in directing larval thermal-induced responses *in vivo*. This study has also broadened our view on the role of TRPA1 in insect thermosensation by reporting for the first time that TRPA1 is involved in thermal plasticity of *An. gambiae* larvae depending on the cultivation temperature. I took part in the design of experiments, data acquisition, statistical analysis, figure and manuscript preparation.

Introduction

Anopheles gambiae sensu stricto (Diptera: Culicidae) is the principal sub-Saharan vector of human malaria that causes over a million deaths annually¹. As is true for all mosquitoes, *An. gambiae* goes through pre-adult development spanning egg, larval and pupal life stages in aqueous environments. This period typically lasts between 5 and 14 days, depending on population density, food level and water temperatures in larval habitats². Although frequently overlooked, it has long been appreciated that a significant degree of vector control is accomplished through regulation of larval populations. Indeed, efficient regional eradication of malaria has been achieved primarily through larvicidal intervention³. In addition, due to their aquatic lifestyle and considerably less complex nervous system, immature *An. gambiae* represents a more tractable stage for the basic study of various physiological and sensory processes⁴. Indeed, previous studies have taken advantage of both simplicity and reproducibility of larval *An. gambiae* to explore the basic principles underlying adult olfactory-driven responses, which also serve as a foundation for further exploration of other aspects of larval sensory biology^{5,6}.

Mosquitoes are poikilotherms and as a result, are incapable of maintaining thermal homeostasis⁷. Consequently, aquatic larvae rely on

their ability to sense and respond to temperature cues for several survival-dependent behaviors in response to local temperature fluctuations. These include the ability to navigate through rapidly changing water temperatures in larval habitats that are alternately exposed to sunlight and darkness during day/night cycles⁸. Therefore, the functional characterization of thermal sensitivity in mosquito larvae would provide insights into these processes as well as potentially inform our understanding of the adult sensory system and facilitate the development of novel approaches that are designed to modulate larval thermosensory behaviors to elicit larvicidal activity.

While the molecular mechanisms underlying thermosensation in *An. gambiae* larvae remain largely unexplored, earlier studies have established the role of *An. gambiae* TRPA1 (hereafter, AgTRPA1), a member of the Transient Receptor Potential family of sensory proteins, in conferring sensitivity of adult peripheral thermosensory pathways to increasing temperatures from 25 to 37°C⁹. This is consistent with studies in other insects suggesting that TRPA1 represents an evolutionarily ancient multimodal channel protein that is responsible for sensing temperatures across the warm and/or hot range¹⁰⁻¹². In order to continue the exploration of peripheral thermosensation and in particular, the role of AgTRPA1 in this context, we now focus on late-stage larvae that

represents a critical developmental window in establishing vectorial capacity of *An. gambiae*. These studies have characterized the causal relationships between ambient temperature and larval behavior and more importantly, identify AgTRPA1 as a narrowly tuned peripheral high temperature sensor in larvae that is crucial for regulating mobility as well as thermal preference.

Materials and Methods

Mosquito rearing and larval sorting

An. gambiae sensu stricto, originated from Suakoko, Liberia, was reared as described¹³ with modifications for human blood meals described as follows: Five-day old females were allowed to feed on human blood (purchased from Bioeclamation Inc.) for 60 minutes using a Hemotek membrane feeding system (Discovery Workshops, UK) augmented with CO₂ and human foot odors (derived from a well-worn and unwashed athletic sock), following the guidelines set by Vanderbilt Institutional Animal Care and Use Committee. For behavioral and ablation studies, early 4th or 3rd instar larvae were manually picked out from rearing pan, respectively. Prior to analysis, larvae were rinsed gently with ddH₂O on a

clean metal sieve to remove debris and food residuals and kept in room temperature (24-25°C).

Thermo-electric control module

In order to generate either homogenous heating or linear temperature gradients in behavioral arena that was composed of glass petri dish of 150mm in diameter filled with 100ml of ddH₂O, we fabricated a design based on a similar apparatus from¹⁴. Here, a thin anodized aluminum sheet (12 x 8 x 0.25 inch) was placed on top of two anodized aluminum blocks whose temperatures were adjusted by using both liquid-cooling achieved via water blocks (Custom Thermoelectric) connected to a cycling cold-water bath as well as Peltier devices (Swiftech Inc.) coupled with PID controllers (Oven Industry Inc.). Temperature across the aluminum sheet was set using software (MR001 Ver. Rev B, Oven Industry Inc.). Heating/cooling of each Peltier device was monitored in real-time by dual-mounted thermal probes (Oven Industry Inc.) installed on each end.

Fluorescent in situ hybridization and Fluorescent immunohistochemistry on whole-mount larval antennae

Protocol for FISH studies was adapted and modified from¹⁵. Briefly, whole larval antennae from 4th instar stage were hand-dissected into 4% PFA in PBS with 0.1% Triton X-100. Samples were then gently transferred into Pyrex glass dish where all subsequent treatments took place. Pre-hybridization and hybridization were performed under 55° C for 6 and 24h, respectively. Fast red staining was used to visualize anti-DIG antibody linked to alkaline phosphatase (AP). Riboprobes were acquired from⁹ by amplifying 900bp of *AgTRPA1* coding sequence using PCR primers: Forward: 5'-CTATTCGGCGGCTTCAATAAC-3' as well as Reverse: 5'-TCATTTGCCAATAGATTTGTTGAAGC-3'. RNA probes were labeled with digoxigenin to generate sense and antisense. Anti-horseradish peroxidase (HRP) antibody conjugated to FITC was utilized to mark neuronal axon and dendrites. Additionally, anti-AgORco antibody raised from rabbit was used to distinguish between AgTRPA1-expressing neurons and odorant receptor neurons (ORNs). AgORco labeling was visualized by incubation with Alexa Fluor goat-anti-rabbit 488 (Invitrogen). Whole antennae were mounted in Vectashield (Vector Laboratories) and observed with an LSM510 inverted confocal microscope (Carl Zeiss).

Automatic larval tracking and analysis

A digital video camera connected to Ethovision XT tracking system (Noldus Inc.) was used to automatically capture and track locomotion of an individual larva in the glass petri dish. For each trial, a single larva was gently introduced at the center of the arena and given 15s to adapt prior to the onset of recording at 10 frames per second (fps). Locomotion was recorded for a total of 300s. For each temperature setting, a minimum of 15 trials (across an equal number of different individuals) was acquired and parameters such as total distance travelled were calculated using Ethovision software. For antennae as well as palp ablation studies, all manipulations were carried out by manual dissection at 3rd instar stages, after which larvae were allowed to recover for 24h prior to behavioral testing. To quantify thermal preferences, we recorded the time interval that each larva spent in either warm or cold half of a gradient that is expressed as thermotactic index (T.I) and calculated as follows: $(t^{\text{warm}} - t^{\text{cold}}) / (t^{\text{warm}} + t^{\text{cold}})$. A negative index value reflects a situation where larvae are more inclined to stay in the cold half of the gradient (negative thermotaxis) whereas a positive value is indicative of the opposite. For statistical analysis, the comparison of two groups was carried out using Mann–Whitney *U* tests while comparison of multiple groups was achieved using

Kruskal-Wallis one-way analysis of variance. $p < 0.05$ was considered significantly different.

siRNA injection and quantitative RT-PCR

Larval injections were carried out as previously described⁵. 27.6nL of 20 μ M/L siRNA that target 6th and 10th exon of AgTRPA1 coding region (UAAUUGUUGAGCGGAGUGCCAGUU, UUUUUUCUCAUUCGGAUACUCGUU) (Thermo Fisher Inc.) were injected into dorsal side of larval thorax using Nanoliter 2000 systems (World Precision Instruments). Injected larvae were allowed to recover in 27 or 30°C with food provided for 48h. Quantitative RT-PCR was performed to verify the quality of gene knockdown. Ribosomal protein S7 (*rps7*) was chosen as internal control and primers used for these genes were: *rps7*: Forward: 5'-GGTGCACCTGGATAAGAACCA-3' Reverse: 5'-GTTCTCTGGGAATTCGAACG-3' (Amplicon size: 112bp) and *agtrpa1*: Forward: 5'-TATTCGGCGGCTTCAATAAC-3' Reverse: 5'-GCGTTTGAAGGATTTCCAGA-3' (Amplicon size: 115bp). PFAFFL method was used to quantify the relative transcript abundance.

Results

Kinetic larval response to ambient temperatures

In order to understand the molecular processes by which mosquito larvae sense external thermal signals, we first investigated the impact of ambient temperature on larval locomotion. To accomplish this we assayed overall larval mobility as a mechanism to assess larval responses to a range of increasing water temperatures. We obtained uniform heating conditions by programming two Peltier devices to the same temperature set point (See methods). In this manner we were able to precisely control the water temperature within a glass petri dish that was placed upon the aluminum sheet, as monitored by a digital heat probe (HCC-100A DAGAN Corporation). Individual *An. gambiae* 4th instar larvae (reared at 27°C, see methods) were then introduced at the center point of the arena and allowed to swim at will for 5mins subsequent to a 15s acclimation period.

In these assays (Figure 1), *An. gambiae* larvae exhibited relatively high levels of mobility (total distance > 750mm) in cold temperatures (17-21°C); the level of overall movement gradually decreased as ambient temperatures approach 27°C (total distance: 382.7mm). Further increasing water temperature resulted in larval mobility returning to a moderate level at approximately 30°C (total distance: 580.5mm), and then decreasing again as conditions enter the hot temperature range (33-37°C) (total distance<350mm). Not surprisingly, once the water temperature

reached 39°C, larval locomotion increased significantly (total distance: 655.7mm) although conditions by 41°C no longer supported viability while morbidity and/or mortality was evident after 2-3mins of assaying. These experiments indicate that *An. gambiae* larvae are capable of recognizing and responding to varying ambient temperatures, leading to distinctive kinetic responses.

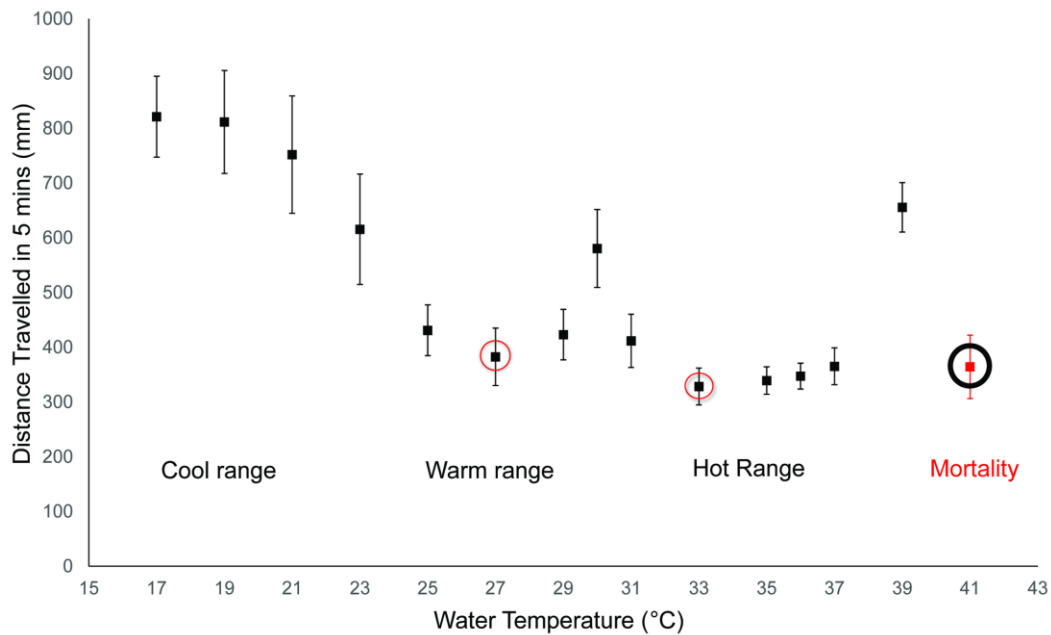


Figure 1. Thermal-induced mobility in WT 4th instar *An. gambiae* larvae. Arithmetic means \pm standard error of the mean (S.E.M) of total distance travelled by individual larva in 300s were plotted ($n \geq 15$). Red circle indicates the two individual temperatures that generated lowest larval mobility in the neighboring temperature ranges (27 and 33°C) while black circle shows the temperature at which larvae experienced morbidity/death after 2-3 mins of assaying (41°C), thus the total distance was calculated based on the time frame before larval mortality.

Thermal-induced kinesis reveals larval thermal preferences

The kinetic responses of *An. gambiae* larvae to individual temperatures are consistent with pre-described patterns of attractive or repulsive stimuli¹⁶. When challenged with a non-directional stimulus such as ambient temperature, faster movements of the subject, or positive orthokinesis, may imply behavioral aversion to the stimulus while slower rates of movement (negative orthokinesis) is consistent with attractive cues¹⁷.

In this light, it is noteworthy that the recorded larval mobility achieved the lowest values at 27°C and 33°C when compared to movement rates at neighboring temperature ranges (17 to 30°C and 30 to 39°C, respectively). This phenomenon raises the hypothesis that *An. gambiae* larvae in this study display a preference for ambient temperatures around 27 and 33°C. To verify this we explored their inherent thermal preferences on a linear temperature gradient (0.67°C/cm). A total of seven gradients were selected for assessment so as to encompass a range of cold (20°C), warm (25, 27, 30°C), hot (33, 35°C) and ultra-hot (40°C) center-point temperatures (Figure 2a). Of these, both thermal gradients across 22-32°C (center point 27°C) and 28-38°C (center point 33°C) failed to induce apparent thermotactic movements in larvae, which spent virtually the same amount of time in

both warm and cool sectors of the arena (TI= -0.03 ± 0.17 and -0.13 ± 0.24 , respectively; Figure 2b). In contrast, larvae displayed positive thermotaxis in gradients with center points at 20 and 25°C (TI= 0.95 ± 0.04 , 0.62 ± 0.18 , respectively) and negative thermotaxis in gradients of 30 and 40°C center point (TI= -0.9 ± 0.04 , -0.91 ± 0.08 , respectively; Figure 2b). Lastly, weak negative thermotaxis was observed in larvae exposed to thermal gradient with center point at 35°C (TI= -0.35 ± 0.22 ; Figure 2b).

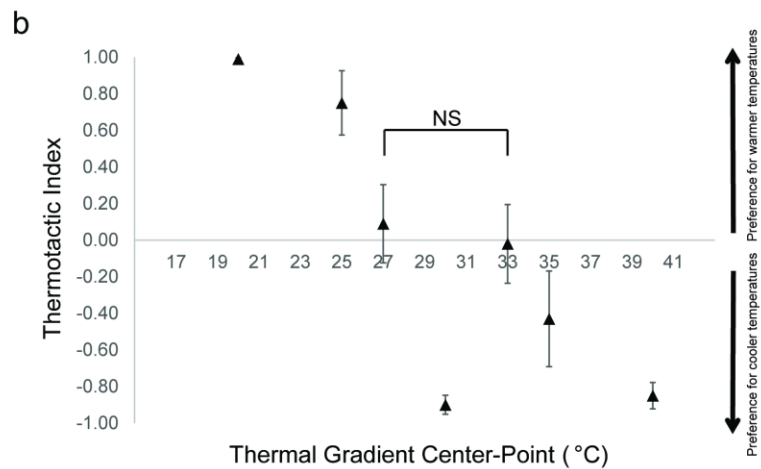
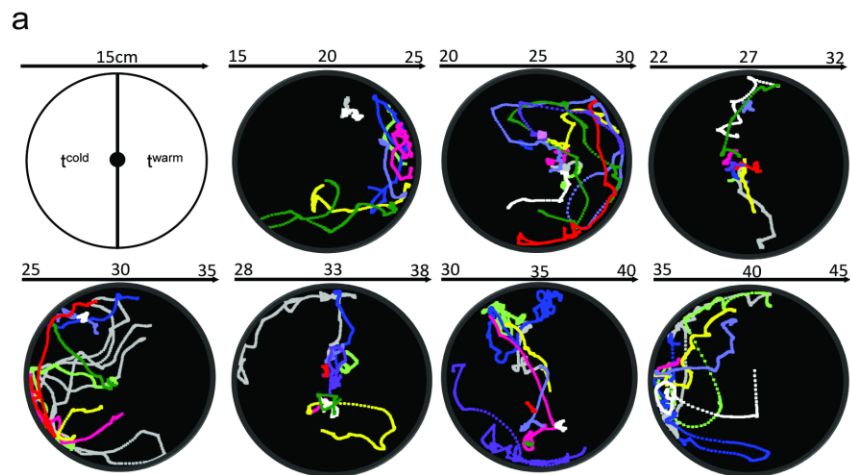


Figure 2. Thermal preferences of WT 4th instar *An.gambiae* larvae.

a) Individual *An. gambiae* larva was introduced into the center of the behavioral arena and recording started following a 15s acclimation period. Swimming trajectories from a minimum of 10 individual larvae reared at 27°C were superimposed. Each color represents a separate trial. b) Arithmetic means \pm S.E.M ($n \geq 10$) of thermotactic indices in 7 different thermal gradients were plotted. Mann–Whitney *U* test was used to compare thermotactic indices at 27 and 33°C with a *p* value > 0.05 .

These data correlate with the larval kinesis at discrete ambient temperatures and suggest *An. gambiae* larvae are capable of distinguishing small variances presented across a linear temperature gradient and moreover, they execute directional movements towards preferred temperatures. Surprisingly, *An. gambiae* larvae display thermal preferences to two distinct temperatures that are 6°C apart (27 and 33°C). It is also notable that cooler half of the gradient was preferred over warmer side when both 27 and 33°C were present in the same gradient (Figure 2a, 25-35°C panel).

Plasticity of thermal-driven behavior elicited by An. gambiae larvae triggered by the shift of cultivation temperature

The observed behavioral preference towards 27°C by *An. gambiae* larvae raises the question as to whether cultivation temperature plays a role in shaping this aspect of thermal preferences since 27°C indeed, coincides with lab rearing conditions. To examine the effect of cultivation

temperature on thermal-driven behavior, we reared larvae at 30°C from eggs obtained from 27°C-colony whilst other rearing conditions (i.e. food, lighting) remained unchanged. Consistent with previous observations, this shift in rearing temperature resulted in no apparent effect other than an increased growth rate such that larvae developed approximately 1 day faster as compared to their counterparts reared at 27°C¹⁸. However, when L4 larvae reared at 30°C were subject to temperature-kinesis paradigm we observed an approximately 3°C shift in larval mobility responses. Here, mobility gradually decreased towards a 30°C trough (total distance: 310.5mm) and then increased to a moderate level at 33°C (total distance: 482.6mm) before undergoing another reduction between 35°C to 37°C, where 36°C represented the second kinesis trough (total distance: 262.3mm). Mobility once again rose at 39° C (total distance 499.5mm) before the onset of larval mortality at 41°C (Figure 3). Additionally, we detected a 3°C upward shift in larval thermotactic indices relative to larvae reared at 27°C as larvae displayed preference to 30 and 36 instead of 27 and 33°C, respectively (Figure 4). These shifts in behavioral responses precisely matched the 3°C rise in cultivation temperature suggesting that *An. gambiae* larvae define their “thermal space” such that the cold, warm, hot temperature sensors are calibrated based, in part, upon rearing conditions. While a subset of these responses appear to exhibit plasticity,

larval behavior within the ultra-hot temperature range (39-41°C) was unaltered by the shift of rearing conditions. This is consistent with the view that aversive responses to noxious temperatures directly associated with lethality would be more rigid.

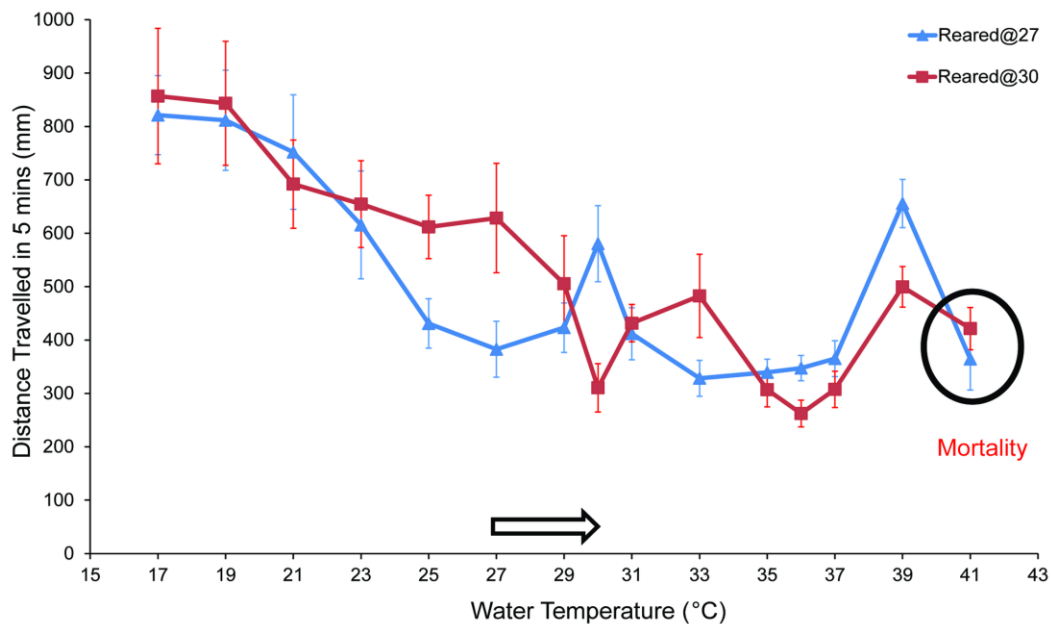


Figure 3. Thermal-induced larval mobility following the shift of cultivation. Arithmetic means \pm S.E.M recorded from larvae reared at both 27 and 30°C of total distance travelled in 300s were plotted ($n \geq 12$). White arrow shows the shift of cultivation temperature from 27 to 30°C. Black circle shows the temperature at which larval mortality was evident for both 27 and 30°C-reared colony. This figure indicates the change of larval mobility pattern matches the shift of rearing temperature.

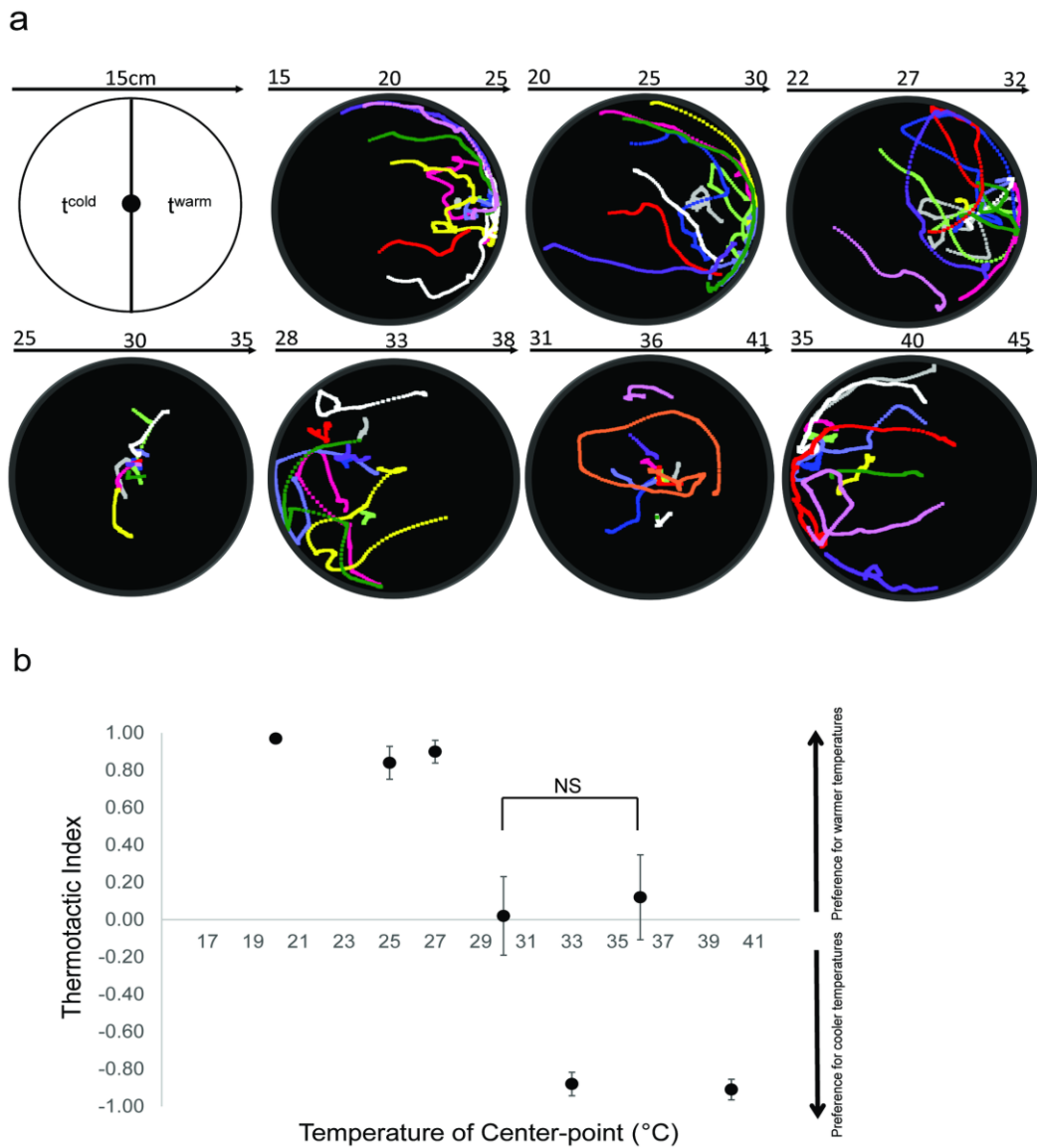


Figure 4. Thermal preferences of WT 4th instar *An. gambiae* larvae following the shift of cultivation.

a) A stack of larval trajectories ($n \geq 10$) recorded in 7 different thermal gradients were shown for larvae reared at 30°C. b) Larval thermotactic indices \pm S.E.M were plotted for larvae reared at 30°C. Mann-Whitney U test was used to compare thermotactic indices at 30 and 36°C with a p value > 0.05 .

AgTRPA1 mediates the larval sensitivity towards hot range temperatures

In light of its role in thermosensory processes in adult stage *An. gambiae*⁹ and other insects¹², it is reasonable to speculate that AgTRPA1 might also play a role in larval thermosensory pathways. To address this we first carried out RT-PCR studies to confirm the expression of AgTRPA1 in cDNA samples isolated from multiple larval tissues including antennae, head and body where AgTRPA1-specific cDNAs were robustly detected in all tissues (Figure 5). Furthermore, whole-mount fluorescent in situ hybridization (FISH) as well as fluorescent immunohistochemistry-based approaches were used to determine the cellular localization of AgTRPA1 mRNA within larval antennae. These studies (Figure 6g–k) revealed a cluster of 14 AgTRPA1-expressing neuronal cell bodies whose dendrites extend apically. As previous studies in *An. gambiae* larvae discovered a morphologically similar cluster of 12 bi-polar olfactory receptor neuron (ORN) cell bodies, we used a polyclonal antisera against the *An. gambiae* odorant receptor co-receptor (AgOrco) which labels all ORNs^{6,19} to distinguish putative thermosensory neurons from ORNs. These studies (Figure 6k–n) demonstrate that the AgTRPA1-positive neurons do not overlay or co-localize with the more distal ORN cell cluster on the larval antennae.

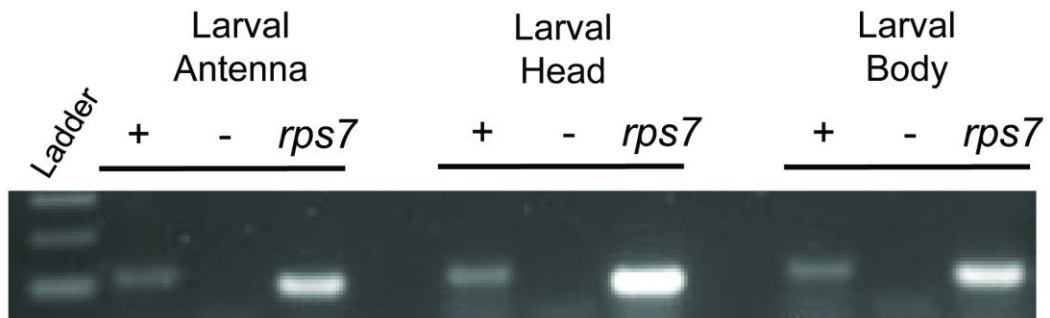


Figure 5. Expression of AgTRPA1 in larval tissues. cDNA libraries from larval antennae, heads and bodies were generated by extracting mRNA followed by *in vitro* reverse transcription. *rps7* and *agtrpa1* were amplified using gene-specific primers and run on a 2% agarose gel. “+” or “-” indicates the presence or absence of reverse transcriptase, respectively.

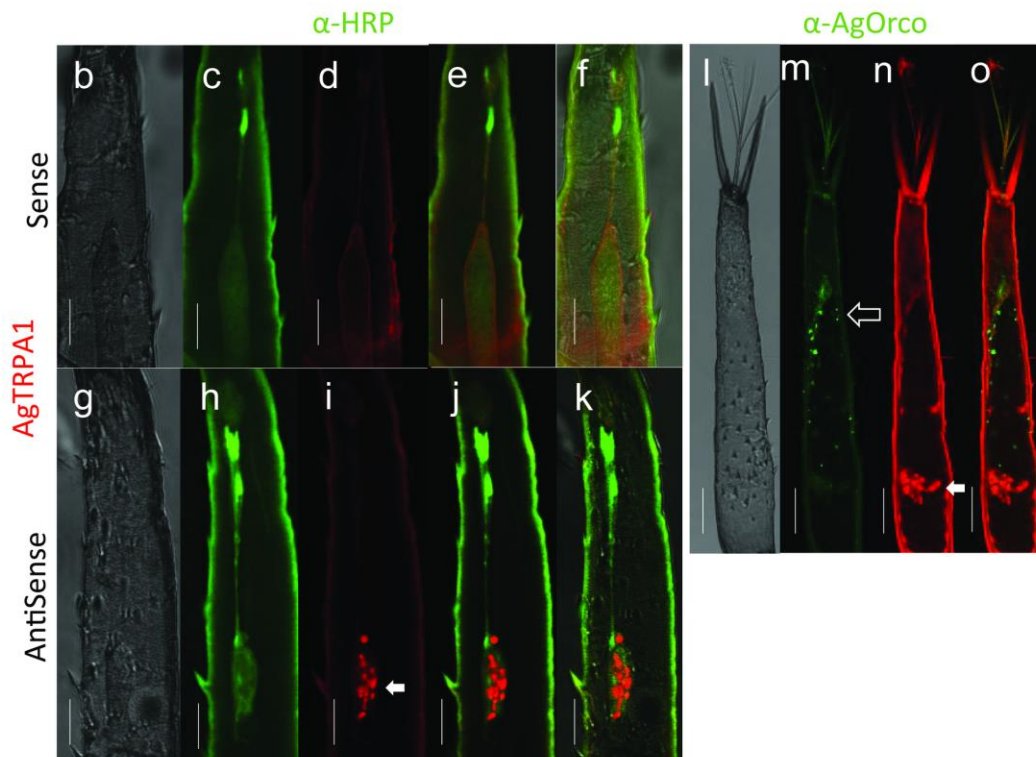
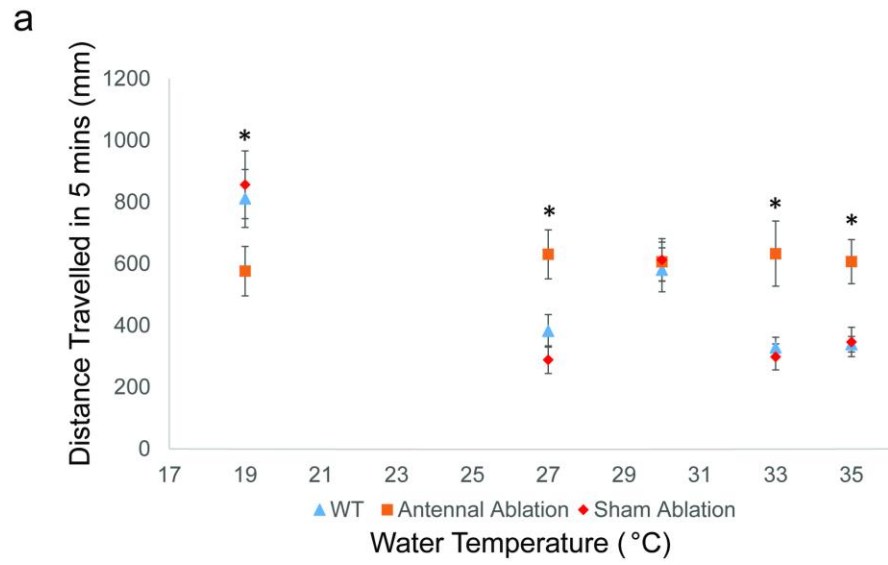


Figure 6. Larval antenna is a peripheral thermosensory organ.

a) Arithmetic means \pm S.E.M of total distance travelled in 300s for individual larva recorded from larvae lacking either antennae or maxillary palp were plotted ($n \geq 12$). Asterisks suggest $p < 0.05$ using Mann–Whitney U test to compare antennal ablation to sham ablation treatment. Kruskal-Wallis one-way analysis of variance was also utilized to compare larval mobility at all 5 temperatures following antennal ablation with $p > 0.05$, indicating larvae without antennae were not capable of eliciting differential mobility at varying ambient temperatures comparing to sham treatment. b–k) Localization of AgTRPA1 mRNA was detected by fluorescent in situ hybridization (FISH). White arrow indicates localization of AgTRPA1 mRNA while green labels neuronal axons and dendrites. l–o) Red fluorescence indicates AgTRPA1 mRNA while green indicates the localization of AgOrco protein that is expressed in all ORNs. White arrow indicates AgTRPA1-expressing neuronal cell bodies while hollow arrow shows cluster of ORNs (Scale bar, 25 μ m).

In order to further assess the potential role of larval antennae in peripheral thermosensory responses, we carried out behavioral assays following ablation of either the antennae or, as a control, the maxillary palps. In temperature-kinesis studies larvae lacking antennae elicited relatively same level of mobility (total distance: 580-620mm) towards five selected water temperatures which were statistically insignificant from each other (19, 27, 30, 33, 35°C) ranging from cold to hot ambient conditions (Figure 6a). However, in larvae receiving a sham treatment these behavioral responses were statistically indistinguishable from unmanipulated group. Taken together, these data are consistent with the hypothesis that the antenna acts as a peripheral thermosensory

appendage that is critical for thermal-induced responses in *An. gambiae* larvae.

Due to the absence of available genetic mutants or a viable methodology to generate gene-specific knockouts, we utilized RNAi-mediated gene-silencing to reduce AgTRPA1 mRNA in order to examine the *in vivo* role of AgTRPA1 in larval thermosensation. Small interfering RNA (siRNA) oligonucleotides targeting AgTRPA1 were injected into L3 larvae along with injection of buffer-alone and a non-specific siRNA targeting a gene (AT5G39360) from *Arabidopsis thaliana* that lacks significant homology to *An. gambiae* genome. Knockdown of AgTRPA1 transcript was assessed using quantitative RT-PCR, which showed on average an 80% reduction of mRNA levels (Figure 7) as compared to non-specific siRNA treatment.

	CT Mean <i>rps7</i>	CT Mean <i>agtrpa1</i>
Non-specific siRNA inject	22.46, 20.42	30.12, 29.95
Buffer inject	21.18, 20.36	28.74, 29.89
AgTRPA1 siRNA inject	16.77, 18.44	27.34, 29.82

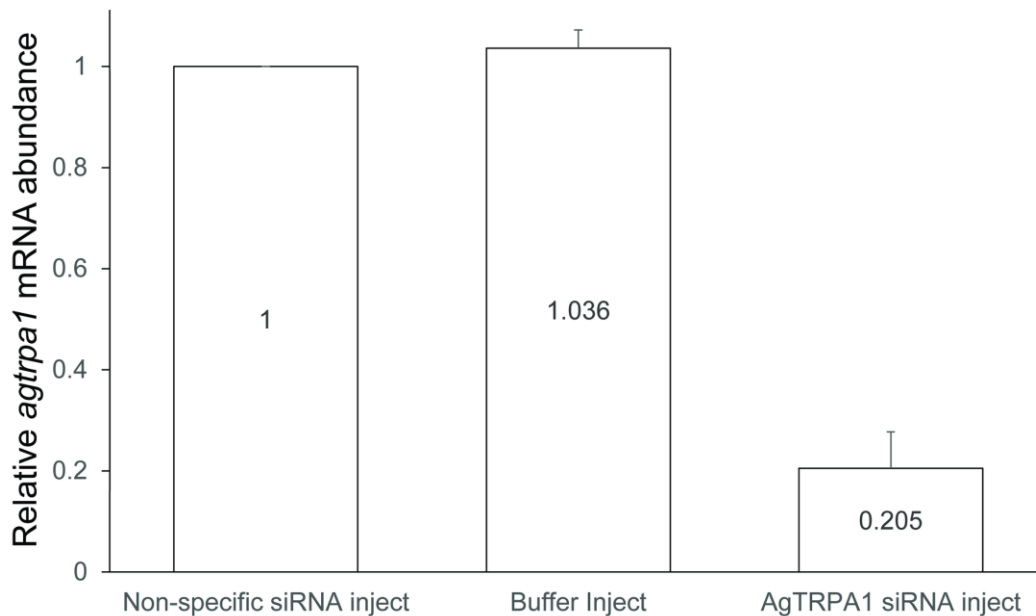


Figure 7. Knockdown of AgTRPA1 mRNA via RNAi. Means of cycle threshold (CT) values for amplification of *agtrpa1* and *rps7* were shown (n=2). Quantitative RT-PCR was performed on cDNA isolated from whole larvae receiving AgTRPA1, non-specific siRNA and buffer injection. Relative mRNA abundance + S.E.M was plotted with data normalized to non-specific siRNA treatment using PFAFFL method.

Behaviorally, *agtrpa1* knockdown gave rise to a selective effect on larval thermosensory responses that was revealed using both mobility and preference paradigms. In these studies, larval mobility was essentially unaffected relative to controls within the low to mid-temperature ranges while mobility within upper range temperatures (33, 35, 36°C) were

significantly increased in AgTRPA1 siRNA-treated larvae (Mann-Whitney U , $p < 0.05$) (Figure 8a). Similarly, *An. gambiae* larvae receiving AgTRPA1 siRNA showed selective alteration of their thermal preference within the same temperature range where thermotactic indices relative to the non-specific siRNA group decreased at 33 (-0.71 ± 0.16) and 35°C (-0.70 ± 0.11), although the effect achieved at 35°C was statistically insignificant (Figure 8b). These data suggest a role for AgTRPA1 as a selective upper range temperature sensor in *An. gambiae* larvae.

Larval behavior in the shifted hot range is also AgTRPA1-mediated

To further validate the *in vivo* role of AgTRPA1 in sensing upper range temperatures, we analyzed thermosensory responses in larvae following a 3°C cultivation shift combined with injection of AgTRPA1 siRNA. In kinesis studies, shifted and siRNA-treated larvae displayed normal mobility reductions at their 30°C cultivation point but significantly elevated mobility at 35, 36, 37°C due to the AgTRPA1 knockdown ($p < 0.05$, Mann Whitney U) (Figure 9a). Similar results were obtained using our thermal gradient assay, where AgTRPA1-dependent hot temperature preference at approximately 6°C above the new 30°C cultivation point was selectively affected by AgTRPA1 silencing whereas larval preferences for

the newly shifted cultivation point was still unaffected by AgTRPA1 silencing (Figure 9b).

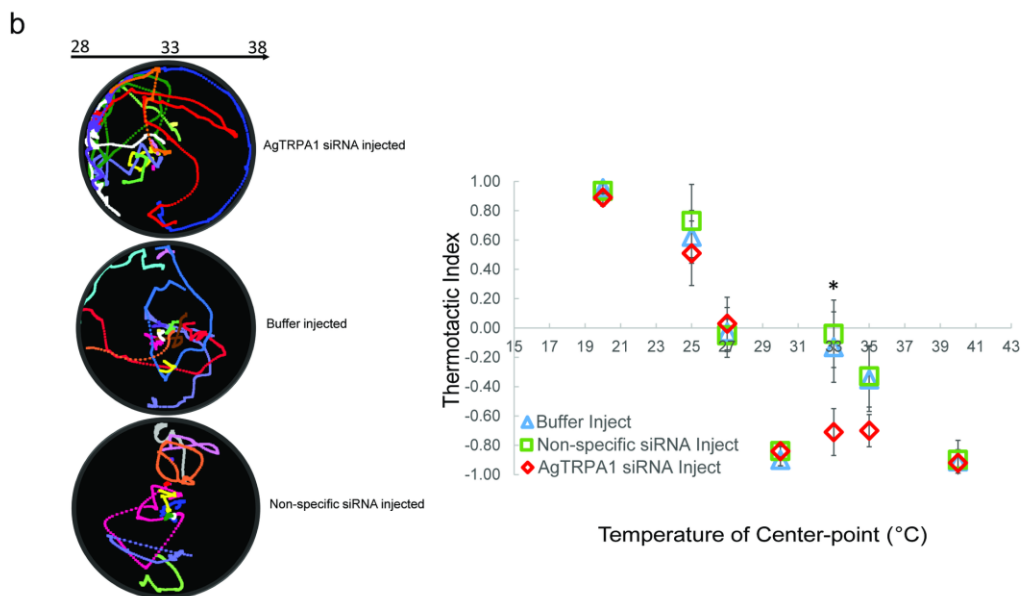
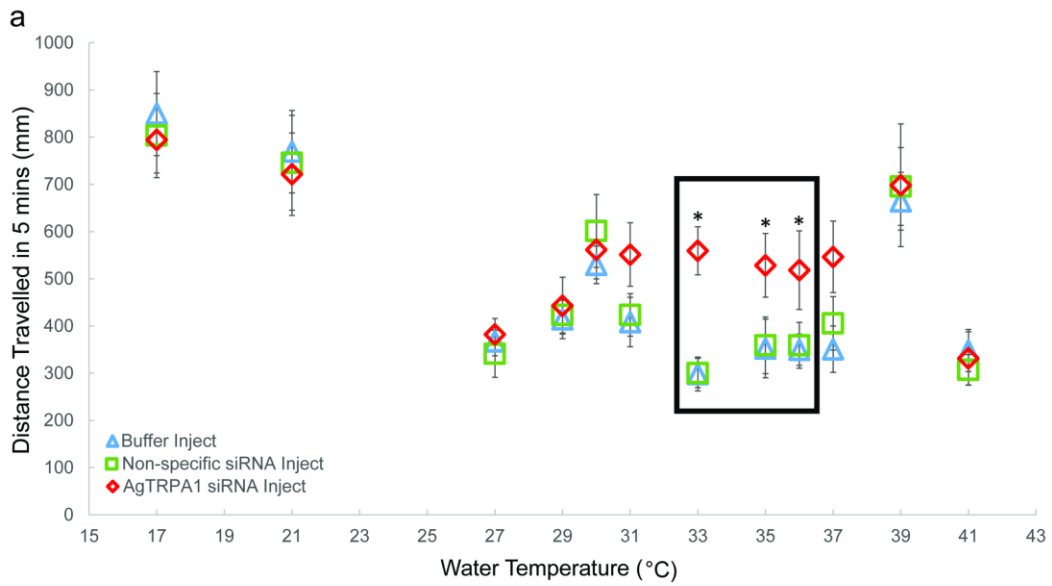


Figure 8. AgTRPA1 mediates larval responses within the upper temperature range.

a) Arithmetic means \pm S.E.M of total distance travelled in 300s for injected larvae reared at 27°C were plotted. Asterisks indicate $p < 0.05$ comparing AgTRPA1 and Non-specific siRNA-treatment using Mann–Whitney *U* test. Black rectangle labels the temperature range at which larval mobility was significantly modified following the knockdown of AgTRPA1. b) A stack of larval trajectories ($n \geq 10$) recorded in 28-38°C gradient for buffer-alone and AgTRPA1, Non-specific siRNA-injected treatments were shown. Thermotactic indices \pm S.E.M were plotted for injected larvae. Asterisks indicate $p < 0.05$ comparing AgTRAP1 and Non-specific siRNA treatment (Mann–Whitney *U* test).

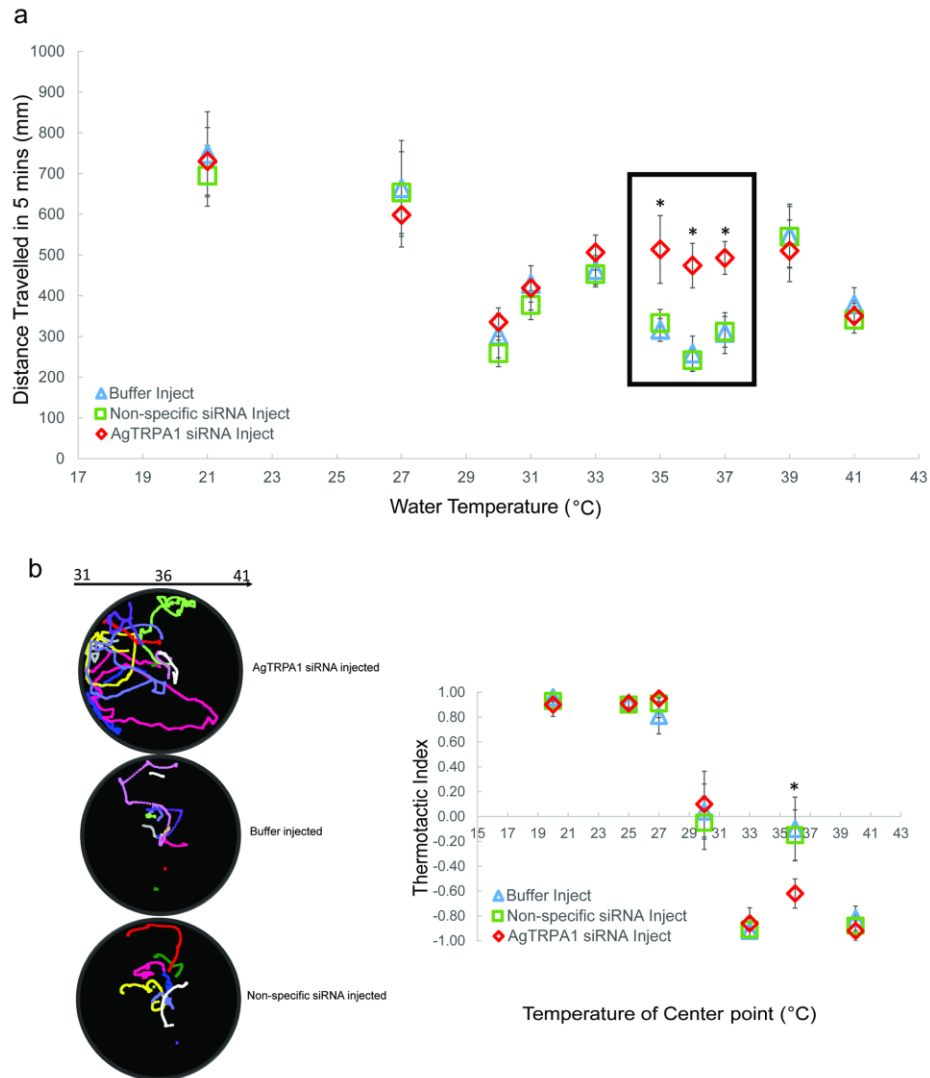


Figure 9. AgTRPA1 mediates larval behavior within the shifted hot range. a) Arithmetic means \pm S.E.M of total distance travelled in 300s for injected larvae reared at 30°C were plotted. Asterisks indicate $p < 0.05$ comparing AgTRAP1 and Non-specific siRNA-injected larvae (Mann–Whitney U test). b) Stack of larval trajectories ($n \geq 10$) recorded in 31-41°C gradient for buffer and AgTRPA1, Non-specific siRNA treatments were shown. Thermotactic indices \pm S.E.M were shown for injected larvae reared at 30°C. Asterisks indicate $p < 0.05$ comparing AgTRPA1 and Non-specific siRNA-injected larvae (Mann–Whitney U test).

Discussion

Together with chemosensory and visual modalities, thermosensory responses of immature *An. gambiae* are necessary for a variety of behaviors pertinent to robust development and survival. Environmental temperature has a major influence on the rate of larval development and, as a result, directly impacts vector populations and malaria transmission²⁰. Ambient temperature also influences the growth of algae and bacteria that are the primary nutrients for *An. gambiae* larvae²¹. Although temperature affects the rate of development, the relationship is not straightforward. Indeed, the production of adult mosquitoes is not directly proportional to the rate of larval development such that temperatures resulting in the fastest growth produce fewer and importantly, smaller adults²². This reflects the balance between developmental rate and the behaviors that mediate larval survival and feeding in order to obtain adequate dietary reserves which are associated with adult longevity, fecundity and vectorial capacity²³.

Accordingly, throughout larval life-stage, *An. gambiae* effectively navigate across fluctuating water temperatures that might otherwise lead to sub-optimal nutrition, reduced growth and death^{22,24}. This capacity is particularly essential for Anopheline larvae in tropical and sub-tropical regions where water temperatures in typical larval habitats with direct sun

exposure (i.e. puddles and mud pit) can vary as much as 20°C through day/night cycles⁸. The critical nature of larval thermosensory behaviors underscores the rationale behind studies to characterize the underlying cellular and molecular mechanisms that may, in turn, provide novel opportunities for the development of cost-effective approaches to disrupt these behaviors.

Late-stage *An. gambiae* larvae are capable of responding to diverse temperatures by exhibiting differential kinesis (Figure 1). In our initial survey of thermosensory responses we noted that larval mobility rates are reduced on two occasions, one of which is a discrete point at 27°C followed by a broader interval between 33–36°C that initiates approximately 6°C higher. In light of studies undertaken in other animal systems, these responses may reasonably be associated with behavioral preference while high mobility rates may be correlated with avoidance. Expanding on these observations using a temperature gradient paradigm (Figure 2), we observed that *An. gambiae* larvae are indeed capable of performing thermotactic movements when the surrounding temperature deviates from these favored condition(s).

It is noteworthy that the robust larval preference to 27°C corresponds to their constantly maintained rearing temperature. This is reminiscent of similar observations in *D. melanogaster* where late-stage

larvae exhibit maximal growth rate and minimal mortality near 24°C²⁵ and show behavioral preference towards this temperature when placed on a linear thermal gradient²⁶. In order to further investigate the effect of cultivation temperature on larval thermosensory behaviors, we shifted the rearing conditions of a sub-population of newly oviposited *An. gambiae* embryos 3°C higher to 30°C and allowed normal development to proceed to late larval instars. Under these conditions we observed a parallel 3°C shift in larval behavior in both kinesis and thermotaxis bioassays (Figures 3 and 4), suggesting that *An. gambiae* larvae utilize their cultivation conditions to set and adjust their thermal sensors to sense ambient temperatures. Cultivation-induced thermosensory plasticity has been extensively investigated as a behavioral paradigm to elucidate the mechanisms of neural plasticity and learning in the nematode *Caenorhabditis elegans*. In these studies *C. elegans* exhibit thermotaxis towards a new temperature following a short cultivation shift²⁷. Similar effects are observed in *D. melanogaster* although alteration of thermal preference required a longer shift of cultivation conditions, typically several days²⁸. While the mechanistic basis as to how recalibration of thermal sensors occurs remains unclear, phenotypic plasticity in thermal-driven behavior is crucial for ectotherms where it likely enables them to better adjust to ecological variations²⁹.

In order to determine the mechanisms for *An. gambiae* larval thermosensory responses, we first carried out antennal ablation on the hypothesis that, as is the case for chemosensation⁶, the molecular sensors that detect ambient temperatures to provide input for directing downstream locomotion would likely be associated with this peripheral appendage. This is supported by our ablation studies which demonstrate that *An. gambiae* larvae lacking antennae fail to discriminate between cold and hot ambient conditions across a range of temperatures whilst interestingly maintaining discrete responses to 30°C (Figure 6). It is evident that while a significant proportion of temperature sensors are antennal, additional and as yet cryptic thermosensory signaling pathway(s) exist.

At a molecular level, and in light of its role as a thermosensory receptor on the adult antennae⁹, we focused on the role of AgTRPA1 in these processes. In larvae, as in adults, AgTRPA1 transcripts are not restricted to the antennae but also detected in head and body tissues (Figure 5). Within the antennae, AgTRPA1 transcripts localize to a discrete set of proximal neurons that are distinct from the more distal group of AgOrco-expressing ORNs that subtend the sensory cone (Figure 6). The segregation of olfactory and thermosensory receptor neurons within the antennae is consistent with other *Diptera*³⁰.

Studies utilizing siRNA-directed specific gene knockdown reveal that AgTRPA1 is required to maintain thermosensory responses to upper temperature range (Figure 8). When heterologously expressed in *Xenopus* oocytes, AgTRPA1 is detectably activated by temperatures as low as 25°C although robust currents are restricted to stimuli above 30°C⁹. This is consistent with *in vivo* effects where AgTRPA1 knockdown results in larvae that respond normally to cold and warm temperatures but show altered kinesis to hot stimuli between 31 to 37°C, although statistical significance is only achieved at 33, 35 and 36°C (Figure 8a). In addition, AgTRPA1 is essential for larval preferences towards this range of ambient temperatures since the silencing of AgTRPA1 decreases the thermotactic indices at 33 and 35°C. This is similar to the thermosensory threshold of TRPA1 in *D. melanogaster* larvae where dTRPA1 is also activated at moderately elevated temperatures ($\geq 30^\circ\text{C}$) although in fruit fly, dTRPA1 is required for thermotactic avoidance³¹.

The conservation of TRPA1-dependent thermosensory discrimination between *Drosophila* and *Anopheles* larvae in the face of dramatic phenotypic divergence in thermal preference is most likely a consequence of their distinctive terrestrial and aquatic ecology, respectively. In addition, crawling *D. melanogaster* larva biases its forward movements with abrupt reorientation or turns in thermotaxis³² while

swimming *An. gambiae* larvae regulate the distance travelled and latency between repetitive “body twisting” maneuvers³³. Signaling cascades may have evolved such that thermal stimulation of TRPA1 leads to differential effects on larval motor neurons. Furthermore, the preferred temperature for a given ectotherm is potentially dynamic in and of itself, changing as a function of developmental, environmental or other factors³².

Larval responses within other thermal ranges, most notably the cultivation point, are not affected by AgTRPA1 silencing and therefore suggest the presence of additional thermal sensors in *An. gambiae*. As is the case in *Drosophila*^{34,35}, it is likely that in *An. gambiae* multiple molecular sensors, each of which function across a discrete temperature range, act together to transduce thermal information that ultimately lead to downstream behavioral responses.

Taken together, these data demonstrate that thermosensory-mediated behavior in upper-range (“hot”) temperatures in larval stage *An. gambiae* is dependent on the function of AgTRPA1. In addition to characterizing these processes in a biologically important system, these studies support the targeting of AgTRPA1 as a viable approach to interfere with larval development and thereby reduce the vectorial capacity of *An. gambiae*. Natural products such as mustard and horseradish that contain allyl isothiocyanate or cinnamaldehyde, both of which act as

potent TRPA1 agonists³⁶, might be used to develop novel approaches to reduce and/or compromise larval populations of *An. gambiae* and, in doing so, the transmission of human malaria.

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Author Contributions

Conceived and designed the experiments: CL LJZ. Performed the experiments: CL. Analyzed the data: CL. Contributed reagents/materials/analysis tools: CL. Wrote the manuscript: CL LJZ.

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CHAPTER IV

Odorant Receptor-Mediated Sperm Activation in the Malaria Mosquito, *Anopheles gambiae*

Preface

The following article was under preparation. I was a co-first author along with my coauthors including R. Jason Pitts (co-first), Xiaofan Zhou (co-first), Juan C. Malpartida (fourth author) and Laurence J. Zwiebel (corresponding author). In this paper, I have developed an *in vitro* bioassay for the purpose of measuring the effect of a spectrum of OR agonists in activating flagellar movement in *An. gambiae* spermatozoa, thus functionally validating the non-canonical expression of these *Ors*. In order to probe the localization of ORco, I took a leading role in using RT-PCR as well as immunohistochemistry to confirm the protein expression of ORco along the flagellum of the mature sperm. I share equal contribution in the design of the experiment, data acquisition, statistical analysis, manuscript preparation with R. Jason Pitts and Xiaofan Zhou. I want to specifically thank R. Jason Pitts for initiating this project as well as taking a leading role in figure preparation.

Introduction

To date, studies of odorant receptor (OR) expression and function in mosquitoes and other insects have been limited to adult and larval head structures where the fundamental properties of insect chemosensation continue to be elucidated¹⁻⁶. Unlike their mammalian counterparts which act as G-protein coupled receptors (GPCRs), insect ORs function as heteromeric ion channel complexes of at least two subunits: one is a highly conserved coreceptor (Orco) and the other (ORx) belongs to a group of divergent ligand-specifying partners⁷⁻¹¹. ORco is required for membrane localization of OR complexes while ORx confers the odor tuning properties¹². Within this paradigm, ligands for numerous members of the *An. gambiae* odorant receptor family (*AgOrs*) have been identified¹²⁻¹⁴. Although *AgOrs* are expressed in tissues beyond adult head appendages, studies regarding *AgOr* function in non-olfactory tissues have not, until now, been conducted. While mammalian and insect ORs operate using distinct modes of signal transduction¹¹, these receptors play similar functional roles in olfactory and potentially non-olfactory tissues. One intriguing possibility, based on OR functional expression in mammalian sperm¹⁵⁻¹⁷, is that *AgOrs* act in a similar context to mediate *An. gambiae* spermatozoa responses to endogenous signaling molecules. Indeed, several studies have suggested the existence of signaling

pathways in non-mammalian sperm^{18,19}, including proteomics analyses in *Aedes aegypti* and *D. melanogaster*, although ORs were not identified in those studies. In a striking example of convergent evolution, we now characterize the expression and functionality of a subset of AgOrs in spermatozoa of *An. gambiae* where they act to modulate activation and perhaps orientation, which are critical to male reproductive fitness.

Materials and Methods

Mosquito Rearing

An. gambiae sensu stricto, originally colonized from Suakoko, Liberia was maintained as described² with modifications for human blood meals as follows. Five-day old females were allowed to feed on human blood (Bioreclamation Inc.) for 60 minutes using a Hemotek membrane feeding system (Discovery Workshops, UK), augmented with CO₂ and human foot odor from a worn, unwashed athletic sock.

RNA sequencing

Testes were dissected from sexually mature, unmated or mated males at 4-6 days post-eclosion into Trizol reagent for subsequent total RNA isolation. Messenger RNA was isolated and sample libraries were

CTCTGGTATCGCCGTTCCGGT	and	Reverse:
GATGTTTTTGGCCATATTGCC;	<i>AgOr6:</i>	Forward:
GGTGAGGATATTGTGGAATCGA	and	Reverse:
GGAAGCTTGCAGGATCTGACT;	<i>AgOr8:</i>	Forward:
AACAAGCTCATCGTTGCGAGCT	and	Reverse:
GAACGAGGTGCTTAGTATCTTC;	<i>AgOr22:</i>	Forward:
GAGTTCAGTACCGGCGTGT	and	Reverse:
GCAGTTTTTCATAACCGCTGT;	<i>AgOr34:</i>	Forward:
TGATGTACGATGAGAGCTTGA	and	Reverse:
CGAGAAACATTTGCACGCTT;	<i>AgOr37:</i>	Forward:
CCATGGAAAAGTGCAACGGATG	and	Reverse:
CATTGCCGAAGCGACATGGT;	<i>AgOr47:</i>	Forward:
CGAACTGACTTTGAAGGGTCT	and	Reverse:
CTAGAAAATGTCCTTCAGCAG;	<i>AgOr70:</i>	Forward:
CGAACAAAAGATTGACGCAATG	and	Reverse:

GACGCTTCAACACACTCATG. PCR amplicons were cloned into plasmid pCRII™ (Life Technologies, Inc.) and sequenced to confirm their identities.

Localization of AgORco

Cryosections of paraformaldehyde-fixed *An. gambiae* testes were collected on glass slides and dried. Slides were processed according to a

previously published protocol² and used as substrates for immunohistochemistry with an ORco-specific antibody²². Primary antibodies were diluted 1:500 in phosphate buffered saline with 0.1% triton X-100 detergent and 5% normal goat serum (PBSTx/NGS). α -tubulin antibody (Genetex, Inc. Cat# GTX628802) was used at a 1:500 dilution in PBSTx/NGS. A custom synthesized Orco peptide (^NHWYDGSEEAKT^C; Selleck, Inc.) was used at a concentration of 1 μ g/ml in a 1:250 dilution with primary antiserum in PBSTx/NGS. A custom synthesized AgOr18 peptide was used at a concentration of 1 μ g/ml in a 1:250 dilution of primary antiserum. Goat anti-rabbit, cyanine 2 conjugated secondary antibody (Jackson Immuno Research, Inc.), was used at a 1:250 dilution in PBSTx. The nucleic acid stain, propidium iodide (Sigma-Aldrich, Inc. Cat# P4864 [1mg/ml]) was diluted 1:1000 in PBSTx with secondary antibodies. A Zeiss LSM 510 (Vanderbilt Cell Imaging Shared Resource) confocal microscope was used to document results.

Spermatozoa bioassay

We developed a bioassay to examine sperm flagellum activation in response to a range of chemical cues. We took advantage of previous AgOr de-orphanization studies that uncovered ligands and modulators for both AgOrco and tuning AgOrs. Briefly, a single testis was isolated from a

sexually mature, 4-6 d.o., *An. gambiae* male and placed in 2 μ l assay buffer (145 mM NaCl, 4 mM KCl, 1 mM MgCl₂, 1.3 mM CaCl₂, 5 mM D-glucose, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.4) containing 10% (v/v) DMSO and test chemical on a clean glass microscope slide (24x50 mm GOLD SEAL, LOT# 121311-9) using a pair of blunt-end forceps to prevent tissue damage. A coverslip (22x22 mm VWR, 040912-9) was placed on top of the preparation and gently pressed 4 times to squeeze open the testis wall and release spermatozoa into the assay buffer (Figure 6). The slide was placed under an inverted microscope equipped with a digital video camera (Ikegami Digital/Zeiss Axiovert 35 at 200X magnification). Videos were recorded for approximately 2 minutes using Ethovision software (Noldus) while the microscope slide was slowly manipulated in the X/Y and focal planes every 10 seconds to scan around the entire testis area (Figure 5B). Each compound and vehicle treatment was repeated 5 to 21 times (average 9) with spermatozoa isolated from different individuals. 8-Bromo-cAMP was obtained from Sigma-Aldrich, Inc. (Cat# B5386). VUAA-class compounds were prepared as previously described²³. Video recorded bioassays were arranged in randomized orders and processed using premier pro software (Adobe Inc.) to remove unnecessary focal adjustment as well as stage moving so that a minimum of 4 fields of view were obtained for

subsequent scoring. Each video clip was viewed by 4 independent observers who were blinded to the treatment conditions and trained to provide a general assessment on the activation level of the spermatozoa by assigning an “activation index” (AI). The qualitative AI scale ranges from 0, no flagella moving, to 3, nearly all flagella moving. All spermatozoa within the field of view were considered. This assay has proven to be very robust and allowed us to rapidly assess sperm responses to chemical treatments. The JMP10™ statistical software package (SAS Institute, Inc.) was used to identify statistically significant differences between mean AIs of test compounds and vehicle, via the non-parametric Mann-Whitney *U* test ($p < 0.01$).

Results

Non-olfactory expression of An. gambiae Odorant Receptor Transcripts

A previous RNA sequencing (RNAseq) study in *An. gambiae* adults revealed that a subset of *AgOrs* are enhanced in whole male bodies in contrast to females among which *AgOrco* showed the highest male-biased expression level between sexes²⁴. One interpretation of those data is that *AgOrs* are expressed in non-head tissues in males where they are utilized in non-canonical chemosensory roles. Given the previous discovery of

functional expression of ORs in mammalian sperm^{17,25,26}, we speculated that *AgOrs* may also be expressed in male reproductive tissues. To address this hypothesis, RNAseq was used to examine relative transcript abundances in *An. gambiae* testes (Figure 1A) where transcripts of more than 30 *AgOrs* were detected; 9 of these *AgOrs* had Reads Per Kilobase per Million (RPKM) values greater than 1 (Figure 1A) and their percentile ranks ranged between 20 and 45. Interestingly, 7 of the 10 most abundant transcripts, *AgOrs* 3, 4, 5, 6, 8, 34, and 37, are predominantly expressed in tissues other than antennae including the maxillary palps, proboscises, and larval antennae^{3,5,6}. Highly correlative results were obtained from age-matched, mated versus unmated testes samples, suggesting that mating itself does not alter *Or* abundance in male testis (Figure 1B). In these studies, *AgOrco* was present at a very low level in one RNAseq sample, and absent in the other (Figure 1A,B). The expression of the 10 most abundant *AgOrs* in testes was confirmed by reverse-transcription followed by polymerase chain reaction, while attempts to amplify *AgOrco* were marginally successful in 2 of 5 biological replicates (Figure 2).

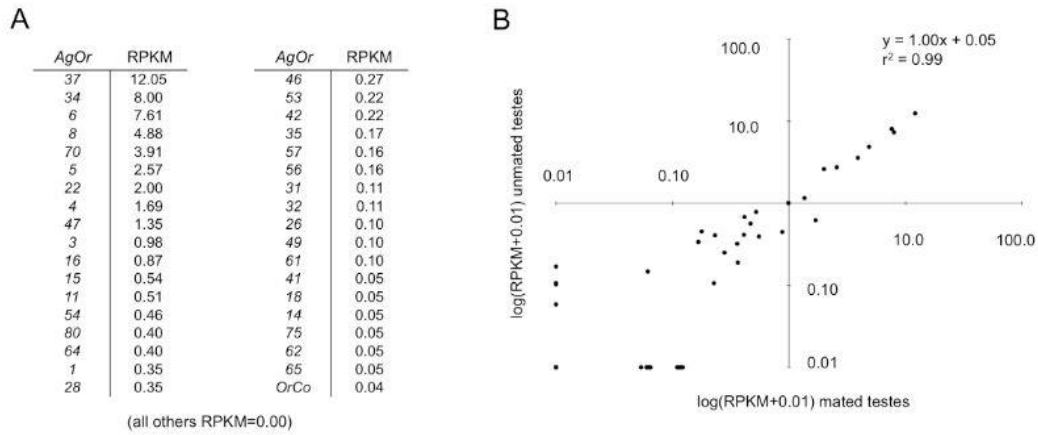


Figure 1. *AgOr* RNA Expression in Testes. (A) Relative transcript abundances of *An. gambiae* odorant receptors in whole testes. RPKM: Reads Per Kilobase per Million reads. (B) Correlation of *AgOr* Transcript Abundances in Testes RNAseq Samples. See also Figure 2.

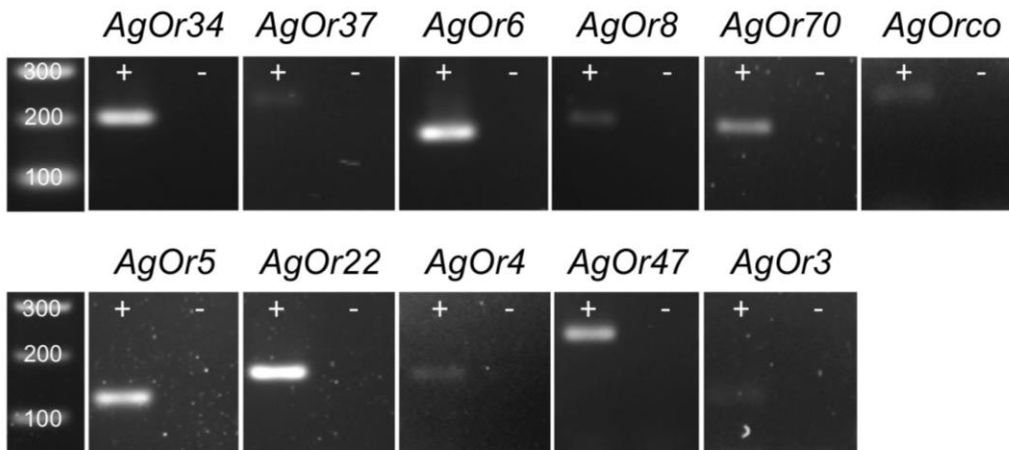


Figure 2. *AgOr* RT-PCR in Testes cDNA. Composite agarose gel images of *AgOr* amplicons derived from testis RT-PCR. Plus reverse transcriptase (+) and minus reverse transcriptase (-) lanes shown. Size markers are 100-300bp.

Expression of AgOrco protein in male reproductive tissues

Detection of *AgOr* transcripts in testes raised the possibility that some *Ors* are expressed as functional proteins in spermatozoa. However, the lack of apparent *AgOrco* transcript might also indicate that *AgOrs* in testes function in a novel manner, one that does not rely on AgORco. Alternatively, AgORco protein may be present, despite the near absence of detectable transcript. To examine this possibility, a previously characterized Orco antibody²², raised against a conserved peptide epitope (see Experimental Procedures) that specifically labels the ORco protein in adult antennae of *An. gambiae* and *D. melanogaster* (Figure 3), was used to probe AgORco protein in testes. In these studies, AgOrco was robustly detected in, including developing sperm cells (Figure 4B,C). Importantly, AgORco labeling was effectively blocked by pretreating antibodies with an ORco antigen-specific peptide, but not with an AgOR18 antigen-specific peptide (Figures 3,5). AgOrco expression revealed punctate labeling along the flagella of mature spermatozoa, coincident with α -tubulin labeling that did not extend into the mid-piece or head region of mature spermatozoa (Figures 4 inset).

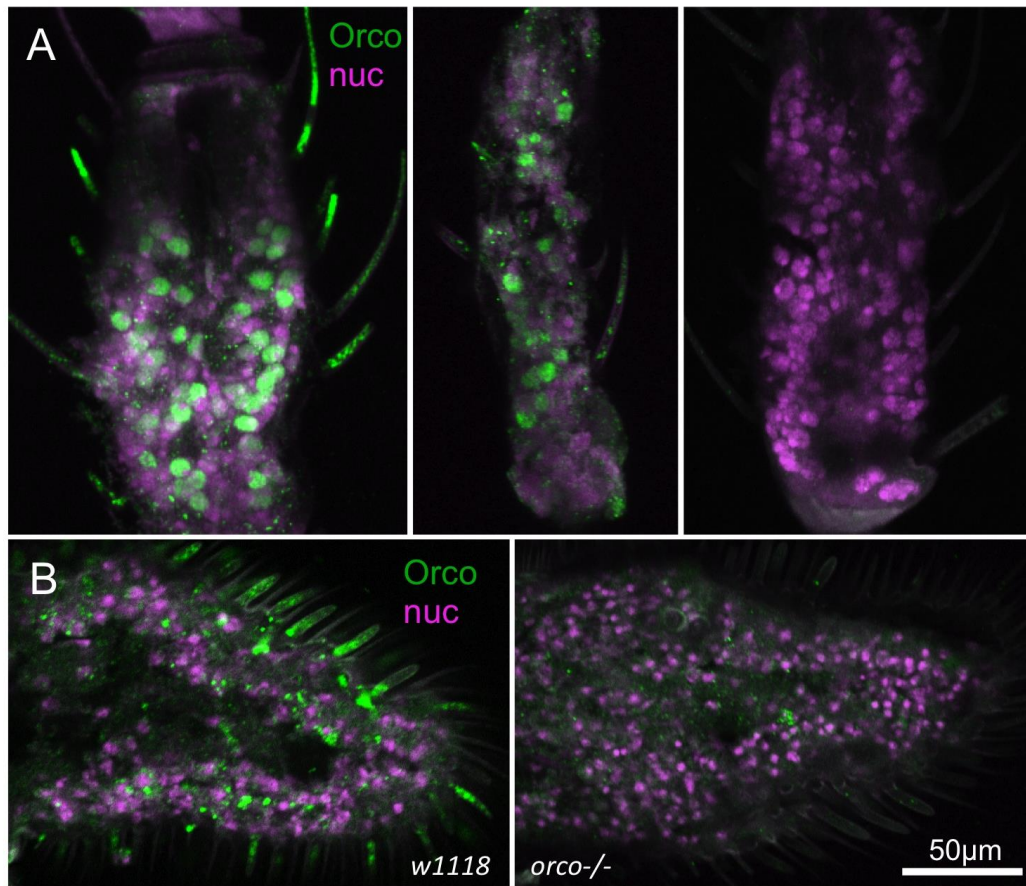


Figure 3. Orco Expression in Antennae.

(A) Left panel: immunolabeling of AgOrco (green) in *An. gambiae* antenna, counterstained with propidium iodide (magenta); middle panel: AgOrco labeling after pretreatment of Orco antibody with an AgOr18-specific peptide; right panel: AgOrco labeling after pretreatment of Orco antibody with an Orco-specific peptide. (B) Left panel: immunolabeling of DmOrco (green) in *D. melanogaster* w^{1118} antenna, counterstained with propidium iodide (magenta); right panel: DmOrco labeling (green) in *orco*^{-/-} mutant antenna. Scale bar applies to all panels.

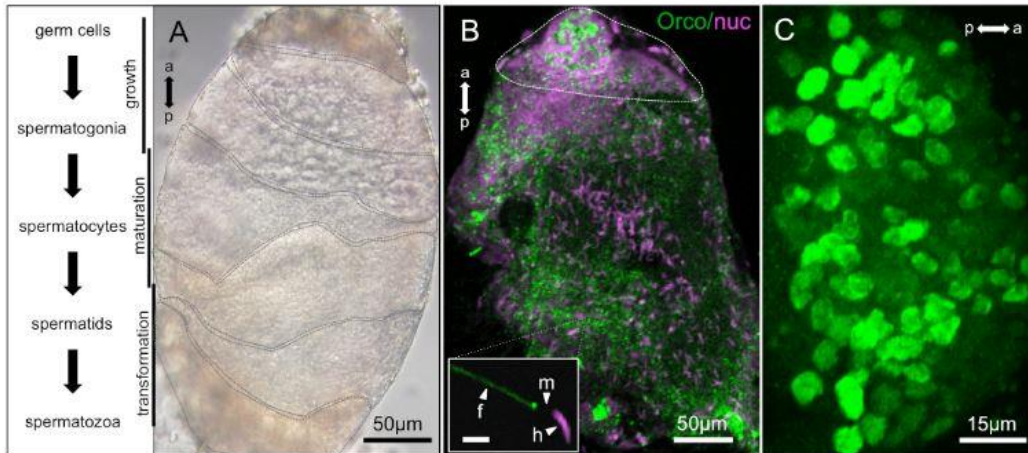


Figure 4. AgORco Protein Expression in Testes.

(A) Differential interference contrast (DIC) image of *An. gambiae* testis showing zones of sperm development. (B) Immunolabeling of AgORco (green) in whole testis counterstained with the nucleic acid dye, propidium iodide (magenta). Germ cell/spermatogonia regions demarcated with dotted line. Inset: high magnification image of single spermatozoa. h-head, m-midpiece, f-flagellum. (C) AgORco (green) in germ cell/spermatogonia region of *An. gambiae* testis. a-anterior, p-posterior.

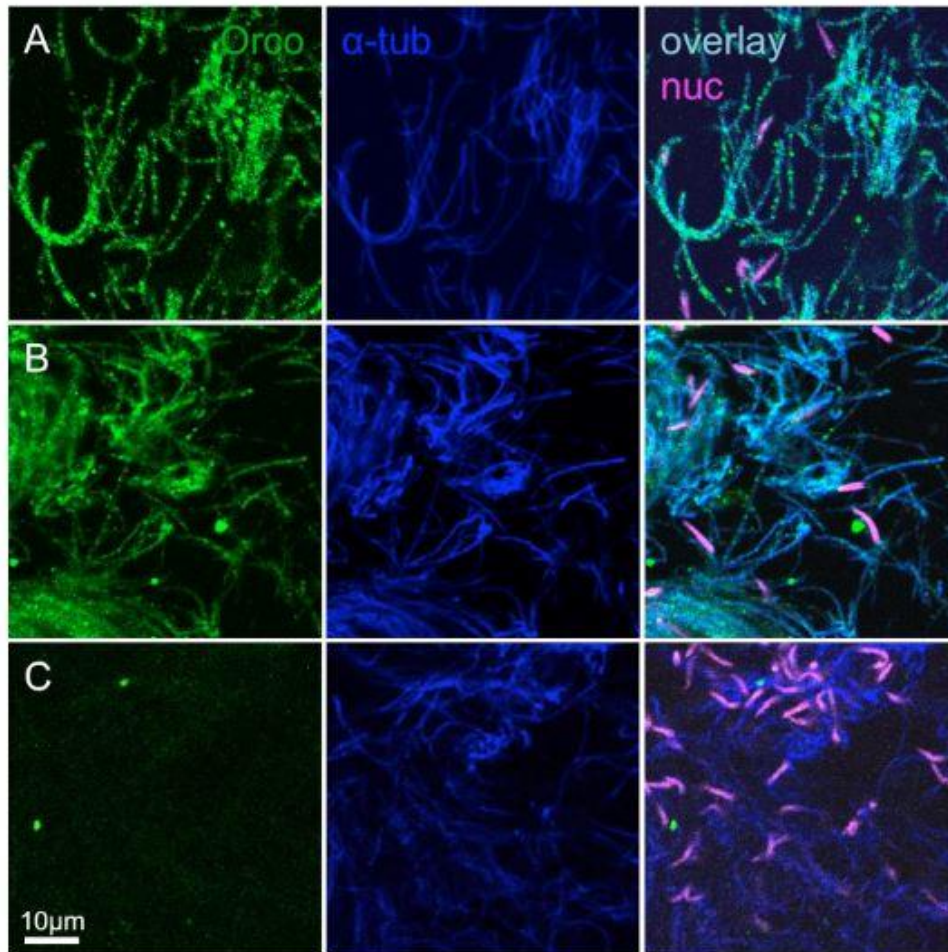


Figure 5. AgORco Expression in Spermatozoa.

(A) Left panel: AgORco (green); middle panel: α -tubulin (blue); right panel: overlay (cyan) with propidium iodide (magenta) in spermatozoa. (B) Left panel: AgORco antibody preincubated with AgOR18 peptide (green); middle panel: α -tubulin (blue); right panel: overlay (cyan) with propidium iodide (magenta). (C) Left panel: AgORco preincubated with ORco peptide (green); middle panel: α -tubulin (blue); right panel: overlay (cyan). Scale bar in (C) applies to all images.

Taken together, these results indicate that transcripts for several tuning *AgOrs* and the *AgORco* protein are expressed in male sperm where they may form functional ligand-gated ion channels. One explanation for the apparent absence of *AgOrco* transcript in testes is that its expression occurs prior to emergence and that the translated protein is very stable, such that it is active throughout the adult male life. Indeed, the apparent persistence of *AgORco* across multiple stages of sperm development is an indication of this stability. These data suggest a potentially unique role for *AgOrs* in *An. gambiae* spermatozoa where they may function as regulators of cell motility in response to endogenous chemical signals.

Activation of spermatozoa

In order to explore the possible biological function of *AgOrs* in *An. gambiae* testes, and in light of the well-established chemosensory responses of vertebrate spermatozoa (reviewed in Kaupp, 2012) we have developed a video-based bioassay (Figure 6) to examine the activation of flagellar beating responses of spermatozoa to a range of chemical stimuli. Because the exaggerated length of *An. gambiae* spermatozoa flagella which can reach means as much as 250 μ m (and up to 2mm subsequent to maturation) as compared to 50 μ m for human sperm²⁷ presents a

technical impediment to isolating individual spermatozoa while maintaining morphological integrity and functional activity, we elected to examine the flagellar beating responses of bulk spermatozoa immediately nascent to ruptured testes.

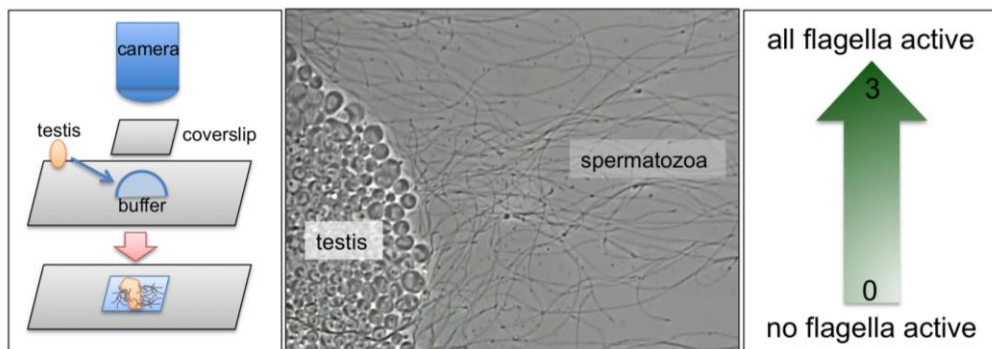


Figure 6. Spermatozoa Activation Assay.

Left panel: testis is removed from live *An. gambiae* male and gently pressed against a glass slide under a coverslip to release individual spermatozoa; middle panel: spermatozoa movements are video recorded and scored by multiple individuals who are blind to treatment conditions; right panel: activation index (AI) scale.

In these assays responses were scored by *post hoc* examination of video clips in a double-blinded fashion (Materials and Methods and supplemental video). These stimulus panels used in these assays were comprised of a range of unitary odorants as well as a set of highly specific ORco modulators that have been recently characterized^{23,28,29}. In these studies, flagella beating responses were significantly elevated in the presence of two ORco agonists VUAA1 and VUAA4, but not in the presence of identical concentrations of a non-potent structural analog, VUAA0 (Figure 7). Moreover, the ORco antagonist, VUANT, did not activate spermatozoa flagella on its own while VUAA1 and VUAA4 agonist responses were significantly reduced when VUANT was co-applied (Figure 7). These robust responses to specific ORco modulators strongly supports the hypothesis that ORco protein is indeed expressed in *An. gambiae* spermatozoa where it forms functional channels, which have the capacity to regulate activation flagellar beating responses.

The presence of tuning *AgOr* transcripts in testes suggests the activation of flagellar beating by ORco agonists could also be mediated by the activity of heteromeric complexes. We therefore speculated that a subset of the known AgOR ligands^{13,14} would also activate sperm flagella to mimicking the effect of VUAA Orco agonists. To examine this we utilized a panel of odorant ligands against testicular AgORs in the

spermatozoa flagella bioassay revealing that exposure to both geranyl acetate and fenchone, which have been shown to elicit responses from AgOrs 11, 31, 35, 56, and 57^{13,14}, indeed induced significant activation of spermatozoa beating (Figure 7). Moreover, the fenchone response was significantly inhibited by the co-application of the Orco antagonist VUANT (Figure 7), indicates that flagellar responses to fenchone are mediated via a functional ORco subunit, supporting the hypothesis that flagellar beating responses of *An. gambiae* sperm involves formation of canonical heteromeric ORco/OR complexes. This represents the first evidence for the function of ORco/OR complexes outside of sensory neurons in *An. gambiae* or, indeed, any other insect.

Interestingly, a membrane permeable form of cyclic adenosine monophosphate (8-Br-cAMP), which together with cGMP are known activators and chemoattractants of mammalian and marine invertebrate sperm^{30,31} also induced a dramatic increase in flagellar movement that was unaffected by VUANT. This suggests the presence of another second messenger-mediated pathway for spermatozoa activation that is either independent or downstream of Orco in *An. gambiae* (Figure 7).

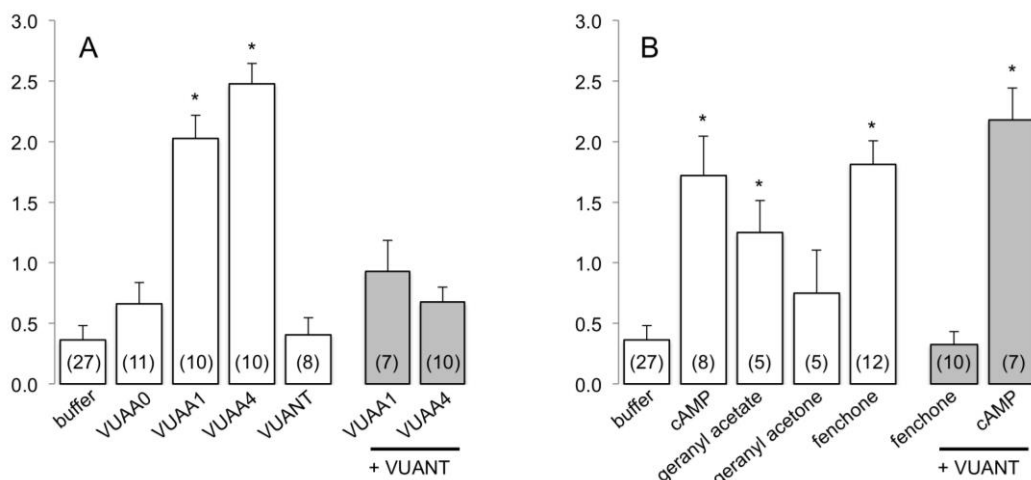


Figure 7. Activation of *An. gambiae* Sperm by Unitary Compounds. (A) Activation indices (y-axis; + SEM) for Orco agonists, VUAA0, VUAA1 and VUAA4 [10^{-3} M] or buffer (white bars). Effect of ORco antagonist, VUANT [10^{-2} M], on agonist activation (gray bars). (B) Activation indices (y-axis; + SEM) for cyclic adenosine monophosphate (cAMP) [10^{-3} M], and unitary compounds (geranyl acetate and geranyl acetone [10^{-3} M], fenchone [10^{-4} M]) (white bars). Effect of VUANT on cAMP and fenchone responses (gray bars). Asterisks indicate significant differences between compound and control buffer samples (Mann-Whitney U , $p < 0.01$). Number of trials for each assay indicated in parentheses. See also Figure S3 and Supplemental video.

Furthermore, the lack of VUANT antagonism of the cAMP activation response also demonstrates that the VUANT reagent is not inherently toxic to *An. gambiae* spermatozoa and that the reductions in VUAA and fenchone-evoked flagella beating responses are specific to their ORco and tuning OR targets, respectively. Not all tested compounds or concentrations elicited flagellar responses as indicated by the lack of

significant spermatozoa activation to geranyl acetone, 10^{-6} M 8-Br-cAMP, 10^{-4} M geranyl acetate and 10^{-6} M fenchone.

Taken together, these data suggest that, heteromeric complexes of AgORs, as well as other signaling pathways, play functional roles in the activation of spermatozoa in *An. gambiae*. Importantly, the role of ORs in sperm activation is likely to be a general feature of insect reproductive biology as immunolocalization studies reveal the presence of the highly conserved ORco protein within spermatozoa of several other holometabolous insects such as the mosquito *Aedes albopictus*, the fruitfly *Drosophila melanogaster*, and the parasitic wasp *Nasonia vitripennis* (Figure 8). We recognize that overt viability and fecundity defects have not been reported for laboratory reared *orco*-mutants in *D. melanogaster*²² and *Ae. aegypti*³², however such conditions do not preclude the presence of a subtle, yet significant OR-based reproductive fitness advantage in natural insect populations. Furthermore, while here we focused on the functional expression of AgORs due to the availability of highly specific chemical modulators, it is also likely that other ion channel and chemosensory receptor gene families may also perform parallel signaling functions in spermatozoa. Indeed, RNAseq-based transcriptome profiling studies consistently revealed transcripts for multiple members of *An. gambiae* variant ionotropic receptor (*AgIr*), gustatory receptor (*AgGr*),

and odorant-binding protein (*AgObp*) gene families are present in the testes of *An. gambiae* males. In total, we found 14 *AgGrs*, 17 *AgIrs*, and 6 *AgObps* that have RPKMs greater than 1, among which 2 *AgGrs*, 4 *AgIrs*, and 5 *AgObps* had transcript abundances above the median of the entire testis transcriptome. These highly expressed chemosensory genes include *AgGr22* which encode the carbon dioxide receptor, and several conserved *AgIrs* with significant antennal expression²⁴.

Discussion

An. gambiae females are generally monandrous and remating is rare in wild populations^{33,34}. This necessitates the storage of sperm in the spermatheca as well as mechanisms for their efficient use over the reproductive life of each female. Few studies have explored the pathways used to identify bioactive substances that elicit responses from conspecific insect sperm and it is likely the volatile AgOR ligands utilized here do not encompass the endogenous signals involved in *An. gambiae* spermatozoa activation. Examples of directed movement of sperm have been extensively characterized in marine invertebrates and mammals³⁵ as well as several insect species. For example, in the beetle, *Drusilla canaliculata*, sperm migrate into the spermathecae³⁶, while the spermathecal gland in the boll weevil, *Anthonomus grandis*, is required for sperm activation,

storage, clearance, and fertility³⁷. In *D. melanogaster*, sperm swim backwards upon entering the female reproductive tract and genetic ablation of the spermatheca secretory cells (SSCs) prior to mating leads to sperm storage defects: sperm fail to migrate into spermathecae and become inactive within the seminal receptacle³⁸⁻⁴¹. Moreover, SSC-ablated females display reduced fertility over time and ovovivipary. These experiments suggest that substances in spermathecae, SSCs, or perhaps other tissues are involved in the activation and chemo attraction of sperm.

Reproductive fitness is an important component in establishing and maintaining insect populations and accordingly, the vectorial capacity of malaria vectors. Despite ongoing efforts to characterize the role of accessory gland proteins and sperm in the formation of the *An. gambiae* mating plug and fertilization⁴²⁻⁴⁷, the potential signals that induce sperm activation, spermatozoa localization, retention or fertilization within the female reproductive tract remain unknown. An intriguing possibility is that females produce and release chemicals that activate male sperm prior to fertilization that also act as chemotactic cues to orient or otherwise direct sperm motility. Importantly, the overall reproductive success of *An. gambiae* males correlates positively with the presence of motile spermatozoa in mated female spermatheca and negatively with sperm length^{27,48}. In this context, an enhanced understanding of *An. gambiae*

sperm activation/motility and the molecular processes that impinge upon them will be significant in terms of both basic biology and as a potential means to develop new control methods.

Existing vector control techniques rely heavily on insecticides; however, insecticide resistance and altered vector behavior following insecticide applications may erode the effectiveness of these technologies⁴⁹⁻⁵¹. Thus, the availability of alternative insect/vector control methods is highly desirable and may ultimately become critical components of integrated pest/vector management programs^{52,53}. One conceptual utility of these studies would be the use of chemical agents, rather than the current reliance on radiation for the induction of male sterility in the context of a Sterile Insect Technique (SIT) program, which has recently been the subject of renewed interest for mosquito control^{54,55}. More detailed investigations of this aspect of the reproductive biology of *An. gambiae* would be essential for the development of novel sterilization or mating disruption technologies that would be expected to significantly reduce vectorial capacity. In addition, the characterization of what is likely to be highly conserved general principles of insect chemoreceptor function in sperm would enhance our understanding of vector biology as well as insect evolution and chemical ecology.

Our findings are reminiscent of the functional expression of ORs in mammalian sperm where signaling machinery with predominant expression and function in sensory tissues is also utilized in reproductive processes. However, inasmuch as mammalian and insect ORs utilize distinct mechanistic paradigms and do not share a common ancestry, OR expression in sperm most likely evolved independently in the two distant lineages, thus providing a striking example of convergent evolution.

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CHAPTER V

SUMMARY AND FUTURE EXPERIMENTS

My thesis work has predominantly focused on the sensory biology of the principal malaria vector mosquito, *An. gambiae*. By designing different apparatuses that were tailored to studying one sensory modality at a time, we have successfully broadened our view on sensory-mediated animal behavior, which is peripherally determined by a plethora of sensory receptor proteins. These studies not only contribute to the understanding of basic biological processes in *An. gambiae*, but also provide insights in the development of novel control strategies that target mosquito populations. More specifically, chapter 2 and 3 study the larval OR and TRP channel-mediated sensory system, respectively, while chapter 4 mainly deals with the reproductive system in adult stage male *An. gambiae*. The central theme that is covered throughout my thesis are odorant receptors and TRP genes, both of which are crucial channel proteins that insects utilize to perceive the outside world in order to engage in a spectrum of behaviors that are vital to the survival of the organism. As for *An. gambiae*, their survival adds another level of medical importance as in order for them to thrive, they have to blood feed on

human hosts, therefore resulting in the transmission of malaria from one infected individual to another with high efficiency.

Numerous studies have suggested that olfaction is the most central sensory modality that mosquito uses for host seeking, mating, nectar feeding, oviposition etc, all of which are life-dependent behaviors¹⁻⁹. If we could elucidate the cellular and molecular mechanisms underlying olfactory-directed behavior, we might be able to interfere/disrupt these processes by specifically targeting the endogenous signaling pathways to reduce malaria burden. Upon the discovery of odorant receptor gene family in mammals which dated back to the year of 1991¹⁰, efforts have been widely taken to demonstrate that the expression of OR proteins within ORN is essential for the animal to recognize ambient volatile odor molecules. The binding of odorant to OR will initiate the conversion of the chemical stimuli to electric action potentials that can be “read” and handled by the central neural system (brain) so as to elicit downstream effects such as learning/memory and behavioral activity (attraction/aversion etc.). However, it is important to mention that OR family is not the only category of receptors that is dedicated to function in the peripheral olfactory system. It is already known that gustatory receptor (GR) in insects is capable of mediating the responses to CO₂¹¹, another volatile “odor”, although GRs are predominantly involved in the contact-

based sensory modality including the sense of touch and taste¹². In addition, more-recent investigations have identified the expression of a novel family of Ionotropic receptors (IRs) in insects that are distantly related to glutamate receptors in mammals which are suggested to respond to odors sharing a non-overlapping odor space with chemicals that are sensed by ORs. These compounds include ammonia and amine compounds¹³, many of which are components in human sweat. Therefore it is indicating that an array of sensory genes from distinct gene families operate in parallel to facilitate insect olfaction. This is also reflective of the complexity of insect olfactory system, considering the “information flow” generated by the enormous amount of olfactory inputs from a spectrum of peripheral sensors.

In chapter 2, we have taken multiple approaches to examine larval peripheral olfactory system *in vivo* by means of functional characterization of both ORs and IRs. We have undertaken studies on larval stage *An. gambiae* for several reasons. Briefly, in this life stage, they typically live in small puddles or lakes and undergo more restricted lifestyle, thereby making them an excellent target for regulation of adult populations. In the meantime, several successes of mosquito eradication were primarily achieved through both larviciding and adult control strategies, demonstrating larval control can serve as a powerful alternative approach

in battling malaria. More importantly, due to their swimming nature, it is much easier to carry out behavioral-related sensory studies on aquatic larvae, whose neural system is much simplified compared to adults. For instance, there are only 12 ORs expressed in larval ORNs while the number in adults is 79, making them a good candidate for basic research. Last but not least, I have successfully developed a protocol for RNAi-mediated gene silencing in larvae, which is still fundamentally difficult to accomplish in adult stage. All these benefits for studying larvae have granted us the opportunity to better probe the role of sensory receptors in leading larval responses *in vivo*. This “model within a non-model system” is especially important to us when genetic manipulations are generally lacking in adult *An. gambiae*. The understanding of larval sensory biology not only will benefit the development of larval control methods, but also has value in terms of the fundamental molecular machinery, which is conserved to a large extent between pre-adult and adult stage mosquitoes.

In chapter 2, we established a robust bioassay to validate the role of OR in sensing both natural and synthetic odors. Furthermore, we identified distinct signaling pathways coupled to either OR or IR by showing *Ag1r76b* was necessary for the larvae to behaviorally respond to butylamine, which was not mediated by OR as knockdown of *Orco*

expression failed to diminish this response. Additionally, we presented for the first time that OR40 contributed to the larval responses to DEET, a commercially available insect repellent that had been widely implemented. Although OR40 is not expressed in the adult olfactory system, this particular finding provides us with insights on the molecular mechanism underlying DEET mode of action, which has been much debated in the field. Interests were given on how DEET repels insects, and it was previously believed DEET played an inhibitory role on insect ORNs, therefore the attraction to human odors was diminished with application of DEET¹⁴. Nevertheless, our study is suggestive of an alternative explanation that instead of masking the effect of other odorants on ORNs, DEET directly binds to a specific OR to provoke repellency. However, this interpretation was questioned based on the broad effectiveness of DEET towards various insect species. It seemed not feasible that each insect expressed a different set of DEET receptor, which was not mutually shared in evolution (OR family is one of the most divergent gene family across insects). However, latest research has favored our model, especially when a group generated *Orco*-mutant *Aedes aegypti* (yellow fever mosquito) and showed they lacked the response to volatile DEET¹⁵ due to the loss of OR-mediated signaling. However, it is worth mentioning that the contact repellency of DEET in the *Orco*-mutant mosquitoes was

retained, which excludes the possibility that OR is the only DEET sensor in adult *Ae. aegypti*, hence additional receptor proteins are also involved. For example, GRs are reported to be indispensable in suppressing feeding behavior in *Drosophila*¹⁶. Taken together, our work has established *in vivo* evidence that both OR and IR-mediated signaling are active in larval peripheral olfactory system. OR40 is the main, if not only, DEET receptor in *An. gambiae* larvae.

Following the analysis of OR functions in peripheral olfaction, in chapter 4, we have indeed, explored a non-canonical aspect of the role played by odorant receptor in the mosquito reproductive system. By means of showing the functional expression of ORs in *An. gambiae* spermatozoa, we put forward a novel model regarding OR function that would renew the contemporary point of view on insect odorant receptors. The binding of odorant molecule to OR is followed by the opening of channel pore and influx of cations. This molecular event in OR gating is probably also conserved in other biological processes that take place in tissues other than the peripheral appendages in mosquitoes. For instance, the activation of flagellar movement in a mature spermatozoa. Mobility is the common feature for sperm cell, with the activation of sperm motion being a prerequisite for fertilization to occur since sperm and egg are physically separated from each other. We started this project in the hope

of identifying endogenous sperm attractant(s) that are produced in the female reproductive organ and show the chemotactic movement of sperm towards the cue(s) is dependent on ORs. However, it was very technically challenging to conduct studies on insect sperm chemotaxis, which was largely due to the difficulty in the isolation of individual sperm. Insect sperm are equipped with extremely long tails (up to 2mm in length), therefore how to overcome the entanglement of sperm tails within a reasonable time frame (sperm need to be viable) would be a key in the tracking/manipulation of sperm behavior. As an alternative, we took a pharmacological approach to monitor the flagellar beating activity of *An. gambiae* sperm in response to a variety of OR modulators. It is very interesting that first, we confirmed the expression of ORs in mosquito sperm and second, the activation of these ORs stimulate flagellar beating.

Our finding is reminiscent of the *in vitro* studies showing OR-mediated sperm chemotaxis in mammals. The vertebrate and invertebrate ORs do not share common ancestry or sequence homology. Not to mention that their modes of actions are very distinct (vertebrate ORs are GPCRs while insect ORs are ligand-gated ion channels). Therefore this work may suggest for a striking example of convergent evolution between mammal and insect ORs. This also adds to the basic biology of OR functions so that a novel target for insect control could be explored.

Specifically, if we can somehow modulate these sperm-expressed ORs render them immobility, this will generate infertile males with no offspring. As a complementary methodology to Sterile Insect Technology (SIT) that is already implemented worldwide to eliminate agricultural pests, this could further decrease the mosquito population. One intriguing possibility, as suggested by our work, could be the use of VUAA-compounds, which specifically agonize/antagonize ORco. Nonetheless, future studies are necessary to fully explore the *in vivo* role of ORs in *An. gambiae* sperm as well as clarify the relationship between OR and reproductive fitness, as we also mentioned in the paper, the lab-reared *Orco*-mutant *Ae. aegypti* are reproducible and do not seem to suffer from fertilization defects. However, even the endogenous function of OR in reproduction is marginal, we could still take advantage of the presence of ORs in mosquito sperm and use them as molecular targets to influence insect fecundity.

In chapter 3, I switched the focus from chemosensory to thermosensory system. This work was inspired by the idea that in addition to the olfactory sensitivity, female *An. gambiae* utilize additional sensory modalities in locating blood meal host. Their sensitivity to the change of ambient temperature would be one example. The combinatorial integration of multiple sensory abilities may increase the efficiency of host seeking behavior. Studying thermosensory processes in mosquito will not only

benefit the malaria research as thermosensation is universal among insects while multiple insect species are reported to be attracted to thermal radiation^{17,18}. Our study in larval thermal sensing has confirmed the role of TRPA1, a multimodal sensory channel protein, in directing locomotion as well as thermotaxis within the upper-range ambient temperatures. This characteristic function of TRPA1 was already well studied in the model insect *Drosophila*^{19,20}. However, we were able to describe a novel feature of TRPA1 in thermosensation by showing the shift of TRPA1-dependent effect following the alteration of larval cultivation temperatures. In the 27°C-reared colony, TRPA1 is essential for the larval behavior in the temperature range of 30 to 37°C with a strong preference to 33°C. Though in the 30°C-reared colony, larval responses in the temperature range of 33 to 37°C is dependent on TRPA1 and the preferred temperature is also shifted to 36°C. It is worth mentioning that larval responses to ultra-high temperature (38 - 41°C) is not affected via the knockdown of TRPA1, thus suggesting the existence of parallel thermosensory pathways. It is likely that thermo-TRPs other than TRPA1 are involved in the perception of the noxious temperatures. Our discovery is hence indicating the antennal expression of TRPA1 could calibrate its mode of action by recognizing the condition at which embryonic development takes place, although the molecular mechanisms are still

elusive. Further studies are necessary to combine the temperature-related learning/memory with the functionality of TRPA1 in *An. gambiae*. Our work on larval thermosensory-driven behavior not only expands the knowledge on the mechanistic aspect of TRPA1 as a temperature-sensing protein, but also considers TRPA1 as a potential target for mosquito control. Natural products such as mustard and horseradish that contain allyl isothiocyanate or cinnamaldehyde, both of which act as potent TRPA1 agonists, might be used in the development of approaches to reduce and/or compromise larval populations.

As a recapitulation, throughout 5 years of my thesis study, I have implemented multidisciplinary practices to functionally examine multiple peripheral sensory proteins that are involved in divergent sensory modalities for the purpose of characterizing their roles in directing downstream responses both at organismal (larvae) as well as cellular level (spermatozoa). I believe this work has broadened our view in insect sensory biology as well as presented novel insights into the future development of mosquito control strategy.

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