NOVEL CHEMORECEPTORS AND GENE EXPRESSION PROFILING IN ANTENNAE AND MAXILLARY PALPS OF *ANOPHELES GAMBIAE*

By

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For my wonderful wife, Lisa, and our beautiful children,

Aaron and Julia, with love.

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TABLE OF CONTENTS

Page

DEDICATION ii

ACKNOWLEDGEMENTS iii

LIST OF TABLES viii

LIST OF FIGURES ix

LIST OF ABBREVIATIONS xii

Chapter

I. INTRODUCTION: MOSQUITOES AS VECTORS OF DISEASE AND THE OLFACTORY BASIS FOR *ANOPHELES GAMBIAE* HOST SEEKING 1

Preface. 1
Introduction 2
Malaria and Mosquitoes 3
Human Feeding Behavior 8
Olfactory Basis of Anthropophily 10
Morphological and Physiological Basis of Olfaction 15
Molecular Basis of Chemosensation 25
Olfactory Signal Transduction 36
Preview of Chapters II, III, and IV 38
References 41

II. DISTINCT OLFACTORY SIGNALING MECHANISMS IN THE MALARIA VECTOR MOSQUITO *ANOPHELES GAMBIAE* 58

Preface. 58
Introduction 58
Methods 61
Mosquito Rearing 61
Individual Larval Behavioral Assays 61

AgIR Identification and Expression 65
siRNA Preparation and Injection 67
Real-Time PCR (qRT-PCR) 68
Results 69
Behavioral Responses of Individual Larva 69
AgORs Silencing Confirms a Direct Role in the DEET Response 73
AgIRs Mediate AgOR Independent Olfactory Responses 82
Discussion 101
References 106

III. TRANSCRIPTOME PROFILING OF CHEMOSENSORY APPENDAGES IN THE MALARIA VECTOR *ANOPHELES GAMBIAE* REVEALS TISSUE- AND SEX-SPECIFIC SIGNATURES OF ODOR CODING 110

Preface 110
Introduction 110
Methods 113
Mosquito Rearing 113
RNA Isolation and Sequencing 113
AgOr and *AgObp* Reannotations 114
Data Processing and Expression Profiling 115
PfamA Categorization 116
Comparison of Tissue Expression Profiles 116
AgOr RT-PCR Amplifications 116

Results and Discussion 117
RNA sequencing and Gene Mapping 117
Gene Expression Profiling in Chemosensory Tissues 122
Chemosensory Gene Families 139
Diverse Roles for Chemosensory Tissues 146
AgOr Expression in Male Bodies 150
Conclusion 151
References 152

IV. OBSERVATIONS ON THE BIONOMICS OF M FORM *ANOPHELES GAMBIAE* FROM A RICE GROWING AREA OF GHANA. 163

Preface 163
Introduction 164
Methods 166
Description of Study Site 167
Dissection 167
Environmental Monitoring 170
Data Analysis 171
Results 171
Discussion 185
References 190

V. SUMMARY AND FUTURE DIRECTIONS 195
Key findings and follow up 195
References 201

LIST OF TABLES

Tables Page

Chapter II

1. Annotation of AgIR family members 87
2. Expression of *AgIr* family members in antennae and maxillary palps. 94

Chapter III

1. *An. gambiae* RNA-seq mapping and expression data 120
2. Enhanced gene classes in *An. gambiae* chemosensory tissues 132

Chapter IV

1. Numbers of the different species collected in the tent-trap and light-trap 173
2. Molecualr forms of *An. gambiae* collected by various methods 174
3. Age structure of the *An. gambiae* dissected from Okyereko 178
4. Age structure of *An. gambiae* that were dead or alive at the time of collection 179
5. Correlation co-efficients at different lags between the different age classes of *An. gambiae* 182

LIST OF FIGURES

Figure Page

Chapter I

1. Worldwide malaria burden 5
2. Distribution of *An. gambiae* in sub-Saharan Africa 9
3. Head appendages of *An. gambiae* adults 17
4. *An. gambiae* antennal sensilla 19
5. Response profiles of *An. gambiae* grooved peg sensilla 23
6. *AgOr* expression in *An. gambiae* chemosensory appendages 28
7. Model of insect olfactory signal transduction 39

Chapter II

1. Operational definitions of larval movements and turns 64
2. Larval responses in *An. gambiae* to yeast and DEET 71
3. Behavioral effects of yeast and DEET on *An. gambiae* 72
4. Larval antennae mediate responses to yeast and DEET 75
5. Quantitative analyses of *AgOr7* and *AgOr40* expression 77
6. Larval response to siRNA-mediated knockdown of *AgOr7* is odorant dependent 79
7. Larval behaviors after injection of non-specific small interfering RNAs 80
8. Larval response to siRNA-mediated knockdown of *AgOr40* is odorant dependent 83
9. AgIR/DmIR phylogenetic tree 89
10. Representative alignments of AgIR and DmIR homologs 91
11. Expression of *AgIrs* in larval antennae 93

12. Expression of *AgIr25a* in adult female antennae 95
13. Behavioral effects of 3MP and butylamine on *An. gambiae* larvae 97
14. Olfactory responses to 3MP and butylamine are mediated by distinct signaling pathways 98
15. Odorant-specific differential effects of *AgOr/AgIr* knockdown 99
16. Quantitative analysis of *AgIr76b* expression 102

Chapter III

1. Read coverage of *An. gambiae* genome 118
2. Gene expression in *An. gambiae* female antenna vs body 121
3. *An. gambiae* enhanced gene pairwise tissue comparisons 123
4. Female antenna vs. palp enhanced gene sets 125
5. Male antenna vs. palp enhanced gene sets 126
6. Female vs. male antenna enhanced gene sets 127
7. Female vs. male palp enhanced gene sets 128
8. Sexual dimorphism in *An. gambiae* chemosensory tissues 134
9. Ammonium transporter genes in *An. gambiae* 138
10. *AgOr* Expression Profile. 140
11. *AgIr* Expression Profile 142
12. *AgGr* Expression Profile 144
13. *AgObp* Expression Profile 147
14. Sensilla-normalized *AgOr* and *AgIr* Expression Profiles 149
15. *AgOr* expression in *An. gambiae* bodies 153
16. *AgOr* expression in *An. gambiae* male reproductive organs 154

Chapter IV

1. Map of Okyereko village 169

2. Numbers of mosquitoes collected Okyereko 2010 176

3. Estimated number of *An. gambiae* by gonotrophic age 180

4. Proportion of newly emerged *An. gambiae* with mating plugs 184

LIST OF ABBREVIATIONS

3MP	3-methylphenol
AgOr	*An. gambiae* Odorant receptor
AgObp	*An. gambiae* Odorant binding protein
AgGr	*An. gambiae* Gustatory receptor
AgIr	*An. gambiae* Ionotropic receptor
CO_2	Carbon Dioxide
cDNA	Complementary Deoxyribonucleic Acid
DEET	N,N-diethyl-3-methylbenzamide
GPCR	G-protein Coupled Receptor
ORN	Olfactory Receptor Neuron
RT-PCR	Reverse-transcriptase Polymerase Chain Reaction
siRNA	small interfering Ribonucleic Acid
SSR	Single Sensillum Recording
TRP	Transient Receptor Potential
UTR	Untranslated Region

CHAPTER I

INTRODUCTION: MOSQUITOES AS VECTORS OF DISEASE AND THE OLFACTORY BASIS FOR *ANOPHELES GAMBIAE* HOST SEEKING

Preface

Despite several decades of global efforts, millions of the world's poorest people continue to be plagued with one or more mosquito-vectored diseases such as malaria, filariasis, yellow and dengue fevers, and numerous other arboviral illnesses. Billions are considered "at risk" of infection in any given year. The reasons for this continuing disease burden are numerous and complex. Among the factors contributing to sustained transmission are the high prevalence of mosquito vectors and often extreme levels of human exposure to them, high holoendemicity of diseases in many locations, agricultural practices that have weakened the effectiveness of insecticides, ignorance among people about modes of transmission and means of prevention, lack of access to basic health needs like clean water, food, and medicines, years of wars and conflict in the poorest regions of the world, and governmental policies that fail to address all of these issues in sustainable ways. Any of these topics are vigorous subjects of debate and intensive study that could easily fill the pages of multiple volumes and are well beyond the focus and scope of this dissertation. It is not the intent of this introduction to cover any of these

topics in detail, but rather to mention them briefly in order to place the work contained herein in a global health context. The ultimate resolution of the issues surrounding disease transmission will require many more years of research and the cooperative efforts of agencies both in the public and private sectors. We have, after all, rich historical examples of places where disease transmission was not only controlled but effectively eliminated, and the courageous people who met those challenges head-on. These successes encourage us to press forward, even in the face of sometimes extreme difficulties. It is my firm belief that humankind will one day witness the elimination of many of these diseases. It is my sincere hope that my own efforts will contribute to, even in small ways, the solution to one of these problems. The following pages offer a brief review of our current state of knowledge regarding mosquito chemosensation, specifically in *Anopheles gambiae*, one of the major carriers of malaria in sub-Sarahan Africa. Moreover, the chapters collectively describe sets of experiments that were designed to increase our understanding of the molecular biology, and to a lesser extent the ecology of, the malaria mosquito. I encourage your participation in this exercise and welcome your comments.

Introduction

Malaria and Mosquitoes

Malaria is a disease that has been known to humankind for many centuries. Hippocrates, writing in the fifth century BC, recorded its symptoms. The debilitating power of malaria has taken the lives of millions of innocent children and brought some of the world's most feared armies to ruin. Alexander the Great is thought to have succumbed to its power (Sherman 2007). Malaria is blamed for much of the slow development of the entire continent of Africa. Remarkably, it was not until the transition of the 19th to 20th centuries when dedicated researchers discovered that mosquitoes were responsible for transmitting the disease. Ronald Ross, a British physician working in India, is given credit for this discovery, and deservedly so, but Carlos Finaly of Cuba is often cited as the first person to suggest that mosquitoes can transmit disease among humans (Guerrant et al. 2006) and others were working on the same premise at the time of Ross' discovery. In the last 100+ years we have learned a great deal about the pathology and treatment of malaria. Despite these advances malaria continues to plague our planet (Figure 1).

Human malaria is a systemic illness that is caused by one of five species of the protozoan genus, *Plasmodium* [*falciparum*, *vivax*, *ovale*, *malariae*, and more recently, *knowlesi* (Sabbatani et al. 2010)]. Many other forms of malaria infect lizards, birds, and other mammals. Malaria is transmitted by mosquitoes of the genus, *Anopheles*. In all, there are about 450 known species of Anopheline mosquitoes, but only about 40 are considered important carriers of human malaria (Hay et al. 2010). The

disease transmission cycle begins when *Plasmodium* parasites (gametocytes) are taken up by a female mosquito in the blood of an infected person. The gametocytes pass through several developmental stages in the mosquito midgut, which includes sexual reproduction. If the mosquito is unable to clear the infection, one or more oocysts may form on the outer surface of the midgut, each producing thousands of sporozoites that migrate through the hemolymph into the salivary glands. From there the sporozoites may be carried along with saliva into the bloodstream of a new host when the infected female mosquito feeds. The sporozoites rapidly penetrate liver cells, develop into merozoites over the course of several days and ultimately leave the liver to invade red blood cells. It is during the blood stage of infection that the clinical manifestations of malaria become evident. Despite differences in some characteristics of the various *Plasmodium* infections, a set of symptoms is shared among them. For example, recurrent fever and chills, excessive sweating, general malaise, muscle aches and headache are malaria's principal manifestations. These signs are often indistinguishable from those occurring with many viral or bacterial diseases. A blood smear is one of the most common and reliable ways to identify *Plasmodium* in active blood stage malaria. *P. falciparum* is the most deadly of human infections, accounting for >90% of malaria deaths worldwide. *P. vivax* and *P. ovale* are generally less severe, but can remain dormant in the liver and produce recurring illness, months or even years after the initial infection.

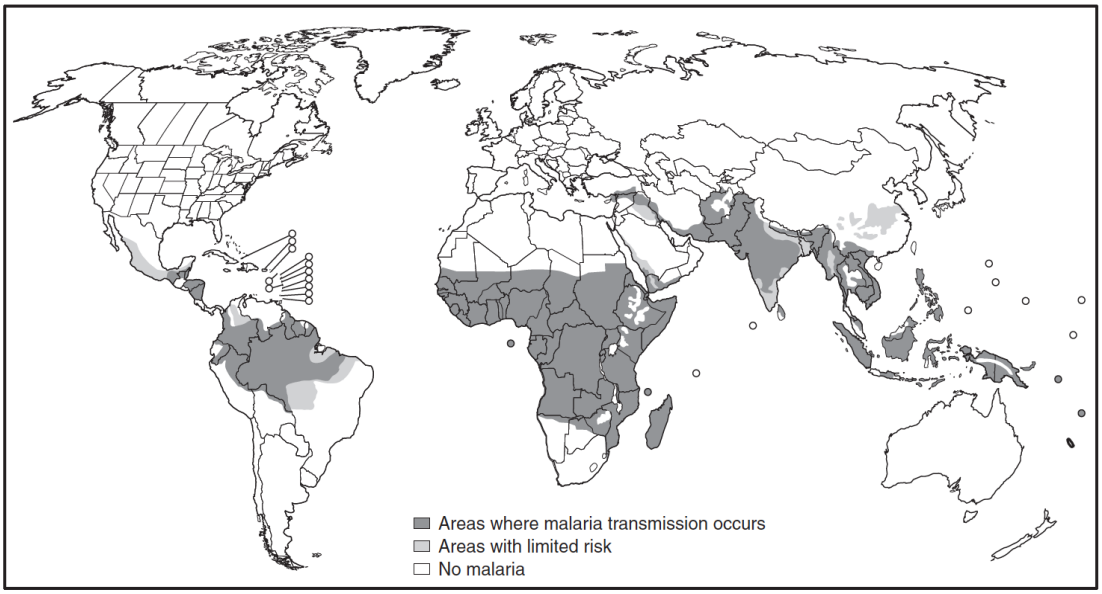


Figure 1: Worldwide malaria burden (Guerrant et al. 2006). Dark shading indicates areas of highest risk.

For an excellent review of malaria see Guerrant et al. (2006).

Malaria's human toll is as difficult to describe as it is to truly comprehend. An estimated 250 million people suffer from malaria each year, and the disease annually kills between 1 million and 2.5 million people, mostly pregnant women and children under 5 (Guerrant et al. 2006). The risks of malaria are not distributed evenly across the globe, with the poorest countries of the world being affected disproportionately. Consider this: annually 58% of malaria deaths occur in the poorest 20% of the world's population and 90% of worldwide malaria cases occur in sub-Saharan Africa (WHO 2003). Despite suffering the greatest consequences, the impoverished are least able to afford effective preventions and treatments and are least likely to have adequate access to them (Barat et al. 2004; Guerrant et al. 2006). The economic costs of malaria are also staggering, both for individuals and for nations. Malaria often reduces family incomes disproportionately across economic classes within a country (Russell 2004). For example, one study estimates that in Ghana malaria reduces the incomes of the wealthiest households by 1% but reduces the incomes of the poorest households by 34% (Sachs and Malany 2002; WHO 2003). At the national level, malaria may reduce gross national products (GNP) of disease-endemic countries by as much as 50% relative to non-malarial countries (Malaney et al. 2004).

Despite the grim picture of the world malaria burden there is reason for cautious optimism. The 2010 World Malaria Report published by the

World Health Organization Global Malaria Program indicates that worldwide malaria cases dropped by nearly 10% during the 10 year period from 2000-2009 (WHO 2010). Moreover the number of reported malaria deaths was down by an astounding 20% during the same period, with many African countries contributing to the decline (WHO 2010). Reductions in African malaria cases coincide with increased intervention programs like Roll Back Malaria (WHO 2002), which worked toward the distribution of Insecticide Treated Bednets (ITNs) that target host-seeking mosquitoes, as well as the introduction of combination drug therapies (WHO 2010). ITNs have proven to be one of the most cost-effective public health interventions, reducing transmission of not only malaria, but other mosquito-vectored diseases (Choi et al. 1995; Nuwaha 2001; Bates et al. 2004; Molyneux and Nantulya 2004; Curtis et al. 2003). Although the RBM campaign has its critics (Yamey 2004), these programs may be at least partially responsible for the gains of the past 10 years. It will be interesting to follow malaria trends over the next decade to see if the improvements of the past 10 years are sustained or even exceeded. Still, the fight against malaria has a long way to go and it is unlikely that these programs alone can eradicate the disease (Yamey 2004; Guerrant et al. 2006). Improving mosquito control measures and continuing to explore innovative ways to reduce human contact with vectors are likely to make important contributions to these efforts.

Human Feeding Behavior

An. gambaie is considered to be one of the most anthropophilic, or "human-loving", mosquito species. Indeed the Human Biting Index (HBI), a measure of the propensity of female mosquitoes to prey on humans, of *An. gambiae* is extremely high, reaching 80-90% in many studies (Tanga et al. 2011; Kasili et al. 2009; Okwa et al. 2009; Garrett-Jones et al. 1980). This preference for human hosts contributes significantly to the ability of *An. gambiae* to transmit disease. However, it is important to understand that most mosquitoes, including Anopheline vectors of malaria, are opportunistic feeders and low HBIs in *An. gambiae* have been recorded, especially in locations where outdoor collections have been utilized or where cattle were present (Kaburi et al. 2009; Muriu et al. 2008; Lardeux et al. 2007; Sousa et al. 2001; Duchemin et al. 2001; Diatta et al. 1998). Conversely, high rates of human feeding have been recorded in mosquitoes like *An. quadriannulatus* and *Culex quinquefasciatus* that are considered strongly zoophilic (Michaelakis et al. 2005; Barr 1967). Anecdotally it is also worth noting that some laboratories maintain colonies of *An. gambiae* by using mice for bloodfeeding. This implies that even highly anthropophilic mosquitoes are at least able to adapt to feeding on non-preferred hosts.

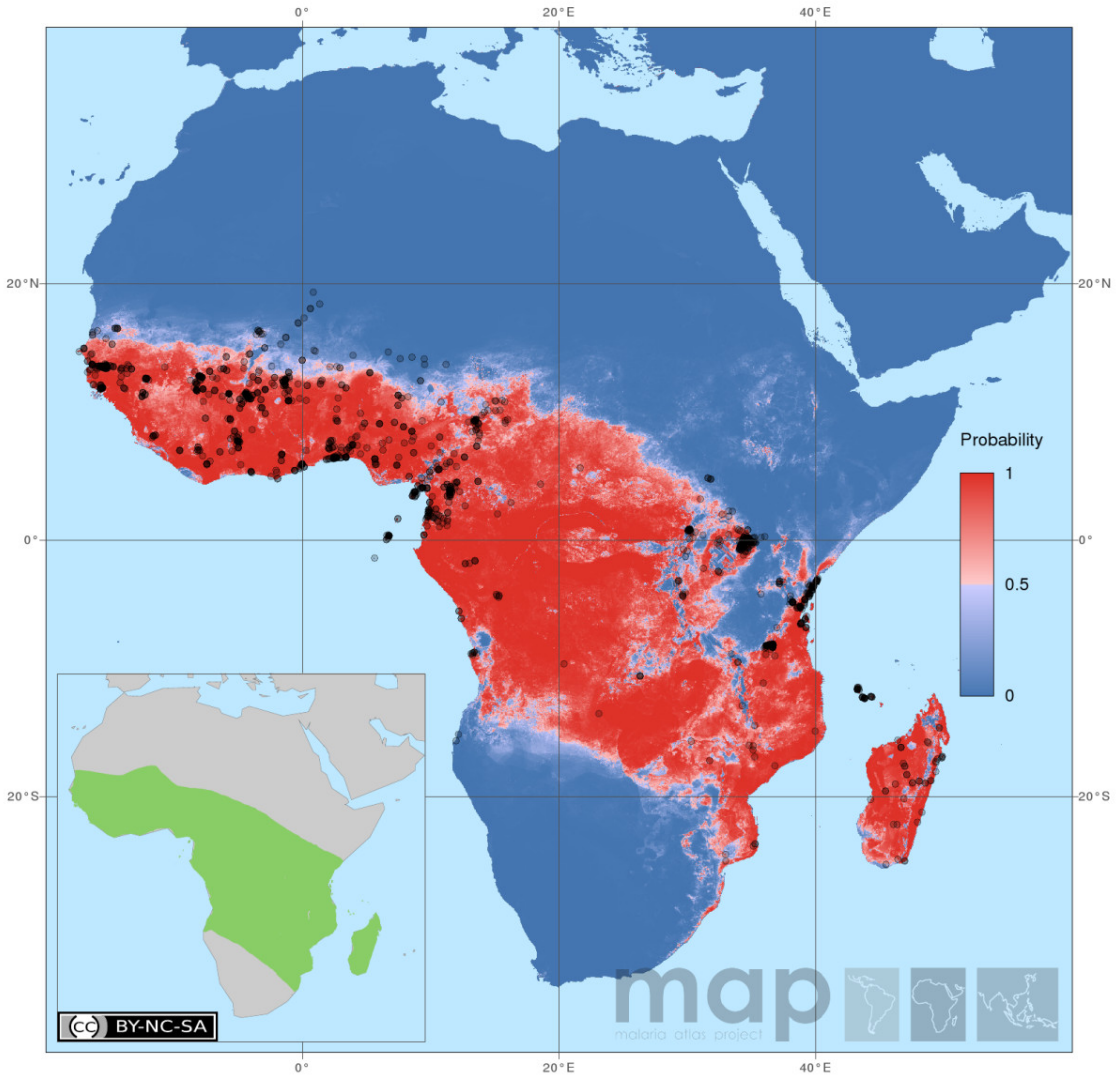


Figure 2. Distribution of *An. gambiae* in sub-Saharan Africa (Sinka et al. 2010). Scale on right indicates lowest (blue) to highest (red) probability of finding breeding populations of *An. gambiae*. Black circles indicate survey sites. Inset shows *An. gambiae* distribution (green) based upon results from expert opinion surveys.

Olfactory Basis of Anthropophily

A multitude of field and laboratory studies have linked odors to host preference in mosquitoes (Dekker et al. 2001; Mboera et al. 1998, 2000; Dekker et al. 1998) and particularly human odors in the case of *An. gambiae* (For reviews see Bock and Cardew 1996; Costantini et al. 1999; Takken and Knols 1999 & 2009; Zwiebel and Takken 2004; Besansky et al. 2004). Strong anthropophily has been demonstrated in choice studies in an olfactometer where *An. gambiae* was significantly more attracted to human odor than was a sibling species *An. quadriannulatus* (Pates et al. 2001a). Moreover, *An. gambiae* showed a strong aversion to cow odor while *An. quadriannulatus* had no preference for either odor source. In a related field experiment, when *An. gambiae* and An. *quadriannulatus* were offered both a human and a calf inside a tent, >90% of *An. gambiae* fed on the human, while *An. quadriannulatus* fed equally on either. In choice studies in South Africa, *An. quadriannulatus* proved to be strongly zoophilic, selecting calf odors or CO_2 preferentially to human odors (Dekker and Takken, 1998). In other field trials, *An. gambiae* has demonstrated a strong preference to human hosts from a distance, even in the absence of visual cues or CO_2, while its sibling species *An. arabiensis* has shown a stronger zoophilic inclination (Costantini et al. 1996, 1998; Mboera et al., 1997). Field collections of mosquitoes using tent traps baited with sleeping humans, again without the possibility of visual contact between the mosquito and host, also catch large numbers

of female mosquitoes (Govella et al 2009). Odor bated traps routinely catch more Anophelines when human odors are used (Njiru et al. 2006), even when competed directly with primate odors (Costantini et al. 2001b). The species composition of the catch depends on local breeding conditions, but often includes a preponderance of *An. gambiae* (Charlwood et al. 2011). These studies and many others (see reviews) have demonstrated that host-seeking mosquitoes are mainly guided by olfactory cues. Sometimes it is unclear whether a given mosquito species prefers one host over another. For example, *Mansonia* spp. showed no preference for human or primate odor in a dual choice field experiment (Costantini et al. 2001b). In those cases, one would necessarily argue for a model of opportunistic host-seeking. While it seems reasonable to argue based on the published research record that *An. gambiae* prefer human hosts, collection methods may have biased some of the outcomes. For example, many studies have utilized indoor resting collections to assess *An. gambiae* host preferences simply because outdoor resting sites are largely unknown or difficult to identify. Furthermore, *An. gambiae* and other malaria vectors are members of species complexes that often live in close sympatry, but occupying perhaps subtly different ecological niches. Therefore what may be true for one member of the complex or even a local ecotype may not be true for all local breeding populations. A recent study has provided evidence for a cryptic subgroup of *An. gambiae* in West Africa by assessing multilocus genetic variation in larvae that were,

by definition, collected outdoors (Riehle et al. 2011). Whether this cryptic subgroup are also anthropohilic is unknown, but the exophilic subgroup was more susceptible to *P. falciparum* infection than sympatric, endophilic *An. gambiae* collected from the same breeding sites. This could have important implications for malaria transmission if one considers that current insecticide programs, using bednets or spraying, target adult mosquitoes that bite or rest indoors. If the endophilic mosquitoes are thus selected against, the exophilic subgroup could be favored, which may paradoxically lead to higher transmission rates. Ultimately it will be important to understand the biting preference of the exophilic subgroup so that mosquito control programs can be more carefully designed. This is an area where novel olfactory-based interventions could serve a critical function by reducing human-vector encounters, regardless of indoor or outdoor resting and biting preferences of the mosquito.

An. gambiae are nocturnal feeders, taking blood meals throughout the night with a peak of blood feeding near midnight, coinciding with locomotor activity (Clements 1999; Wanji et al. 2003). Whether or not *An. gambiae* feed exclusively on humans, one thing is clear: this species reacts very strongly to human odors (Qiu et al. 2004; Zwiebel and Takken 2004). Human odors used as mosquito attractants have been derived from a range of sources including live volunteers, sweat collected from skin, hand and foot rubbings, worn socks, breath emanations, and synthetic blends. Odors have been tested in field traps and in wind tunnels

(olfcatometers). Locating potential a bloodmeal source involves a series of behaviors: activation of insect flight following stimulation with a host chemical odor (kairomone), upwind flight in the direction of the odor, and alighting/probing on the host (Takken 1991). In the context of human odor, approximately 350 different chemical compounds have been identified in sweat (Cork and Park 1996; Bernier et al. 2000). Attractive sweat compounds include carboxylic acids (Meijerink 2001; Meijerink and van Loon 1999; Knols et al. 1997), ammonia (Braks et al. 1999, 2001), lactic acid, and various other volatiles (Meijerink et al. 2001; Healy and Copland 2001; Cork and Park 1996). Moreover, incubated human sweat is more attractive to *An. gambiae* than freshly collected sweat (Braks et al. 2001). Skin microbes are responsible for the changes in chemical composition of sweat that increase its attractiveness (Verhulst et al. 2011, 2010, 2009; Braks 2000). *An. gambiae* females also respond to human breath components, although sometimes based on their repellent effects (Mukabana et al. 2004; Qiu et al. 2010). Another important component of breath is CO_2. While not a human-specific odor, CO_2 has long been recognized as an important mosquito kairomone in field and laboratory settings conditions (Costantini et al. 1996; Gillies 1980; Gillies and Wilkes 1969; Rudolfs 1922). CO_2 acts synergistically to enhance the attraction of other volatiles and is particularly important for the activation phase of host seeking (Lacey and Cardé 2010; Dekker et al. 2005; Dekker et al. 2001; Takken and Knols 1999). Ammonia, like CO_2, acts as a powerful synergist

(Smallegange et al. 2005). Differences in attractiveness to *An. gambiae* among individuals have been well documented (Qiu et al. 2006; Brady et al. 1997; Knols et al. 1995; Lindsay et al. 1993; Schreck et al. 1990). Importantly, a few studies have shown that pregnant women are more attractive to *An. gambiae* or *An. arabiensis* than non-pregnant women (Himeidan et al. 2004; Ansell et al. 2002; Lindsay et al. 2000). Strangely, alcohol intake correlated with higher attractiveness to *An. gambiae* in one study (Lefèvre et al. 2010). The basis for these differences is poorly understood, but highly volatile sweat components are likely the source. Moreover it is apparent that blends of odors and not any single odor constitute a human odor signature that elicits the most profound responses in host-seeking *An. gambiae* females (Smallegange et al. 2011, 2010, 2005; Verhulst et al. 2010; Schmied et al. 2008; Qiu et al. 2010, 2007). Understanding more about the basis of human odor attraction may help us discover new ways to “push” mosquitoes away from human habitations or “pull” them toward baited traps. The idea of a push/pull strategy for mosquito control is based upon a model that has been used effectively in agriculture either to repel crop pests using plant species that are noxious (push) or to attract them using plants that are desirable (pull) but of no commercial value (Cook et al. 2007).

Morphological and Physiological Basis of Olfaction

Mosquitoes are equipped with 3 types of head chemosensory appendages: paired antennae and maxillary palps, and the proboscis, each with distinct sensory functions. In most mosquitoes the antennae and palps are sexually dimorphic while the proboscis is monomorphic (Clements 1999). Electrophysiological responses to host volatiles have been recorded from all three head appendages using sensory physiological techniques. What follows is a summary of the various structural elements that constitute the head chemosensory machinery in *An. gambiae*, which is analogous to many other mosquito species and is broadly similar to many insects from diverse orders. For a good review of many aspects of insect chemosensory biology, consult chapter 10 of **Chemosensory Systems in Mammals, Fishes, and Insects** (Meyerhof and Korsching 2009).

Antennae are attached to the mosquito head by a structure called the scape that serves as an attachment point for muscles that move the antennae (McIver 1982). Distal to the scape is the cup-shaped pedicel that houses the Johnston's Organ (JO), which functions as the mosquito chordotonal (hearing) organ, and is the attachment point for the long segmented flagellum. The flagellum is divided into distinct flagellomeres, numbered 1 thru 13 beginning proximal to the head. Female antennae display chemosensilla on all flagellomeres, while male antennae exhibit long fibrillae (bristles) on flagellomeres 1–11 with chemosensilla restricted

to the distal two flagellomeres (McIver 1982). While the exact role of the fibrillae remains unclear, in *An. gambiae* they become erect prior to swarming and are thought to aid in sensing the wing beat frequency of conspecific females as they fly into swarms of males for the purpose of locating a mate (Charlwood and Jones 1979, 1980; Pennetier et al. 2010). In both sexes the most distal, 13th, flagellomere tapers to a pointed tip, ending in a pair of small sensilla.

Volatile odor perception in mosquitoes, like other insects, is carried out by thousands of primary olfactory receptor neurons (ORNs) that are segregated in groups of 2 or more in small sensory hairs called sensilla. ORN dendrites are bathed in aqueous sensillar lymph contained within porous cuticular extensions (Stocker 2001). Sensilla are classified by both morphology and function. Although sensilla nomenclature often varies between insect species, some general descriptive features are applicable to all. For example, sensilla can be classified as single- or double-walled, thin- or thick-walled, and multiporous or single-pored (Sutcliffe 1994). The single pore sensilla generally mediate contact chemosensation (gustation/taste) and are not considered further here. The reader is encouraged to consult reviews: Scott (2005), Ebbs & Amrein (2007), Yarmolinski (2009), and Montell (2009) on taste perception in insects.

Antennal sensilla have been described for numerous species of mosquitoes, including some Anophelines (Pitts and Zwiebel 2006; Ismail 1964; Coluzzi 1964). Sensilla types are well conserved in mosquitoes,

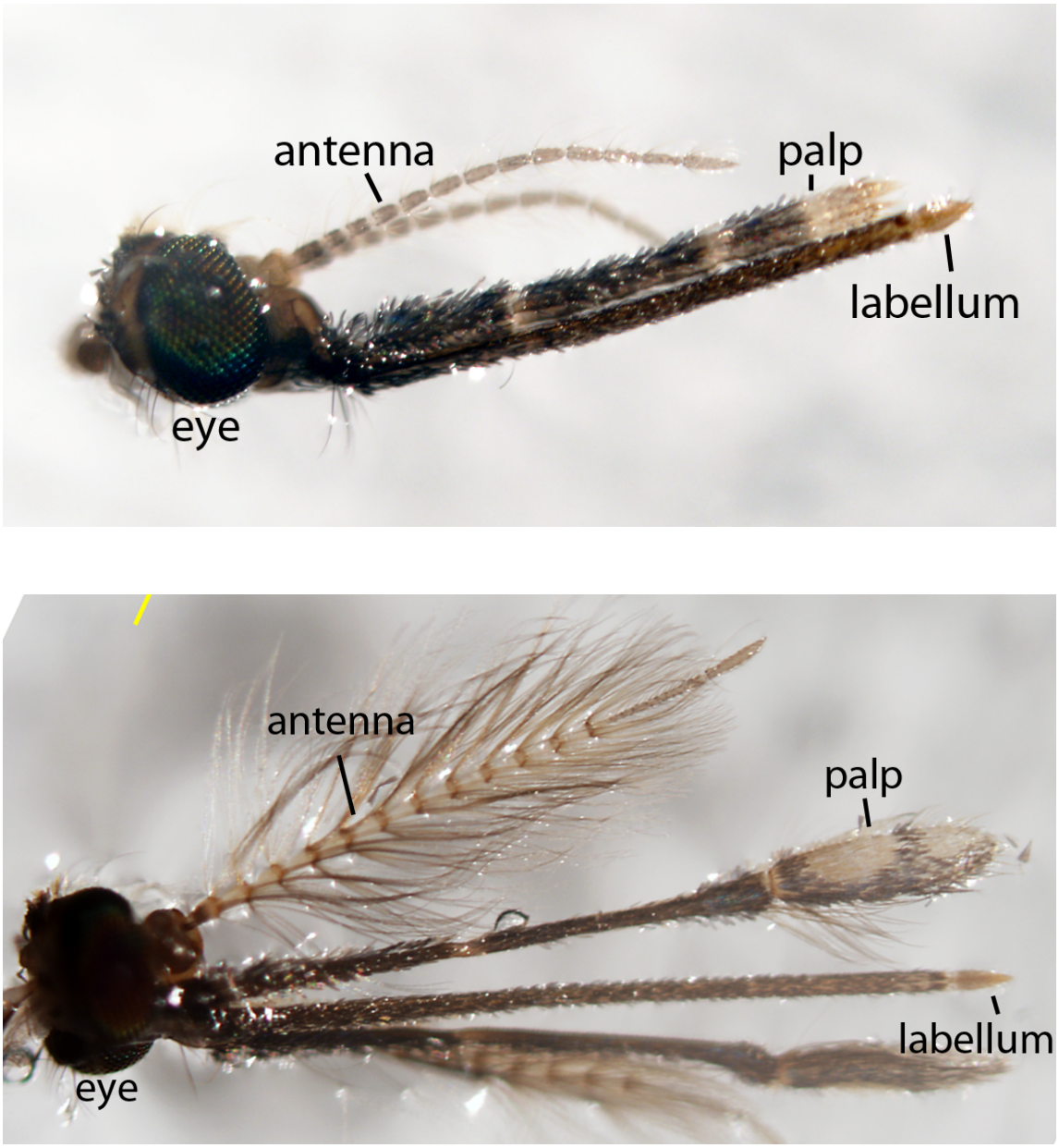


Figure 3. Head appendages of *An. gambiae* adult female (top) and male (bottom), showing sexual dimorphism of antennae and maxillary palps.

although large variations in the numbers of each have been observed.Moreover, female mosquitoes generally have a significantly greater number of chemosensilla than conspecific males, although the types of sensilla are the same in both sexes. Arrayed along the antennal surface in *An. gambiae* are 5 classes of sensilla: chaetica, coeloconica, ampullaceal, trichodea, and basiconica. The sensilla chaetica are sturdy bristles that occur as two distinct subtypes – large and small. Both subtypes are set into sockets at their bases, end in sharply pointed tips and are mechanosensory. Sensilla coeloconica are thick-walled sensilla that occur in large and small forms in the Anophelines. Large sensilla coeloconica are commonly called pitted pegs and are absent in the Culicines. Small sensilla coeloconica also have a peg set into the bottom of a pit and are found paired at the distal tip of the 13th flagellomere as well as in small numbers on 2 other flagellomeres (Pitts and Zwiebel 2006). Little is known about the function of coeloconic sensilla, but the two located at the distal tip of the anteanne are thermosensitive (Wang et al. 2009). Sensilla ampullacea are small, thick-walled peg sensilla set at the bottom of a tube, the external opening of which appears as a very small aperture on the cuticular surface of the first flagellomere. Their small size and location among non-innervated microtrichia made the ampullaceae the most difficult to discern and their function is unknown. Information described in this paragraph was derived from two excellent reviews: Sutcliffe 1994 and McIver 1982, except where noted.

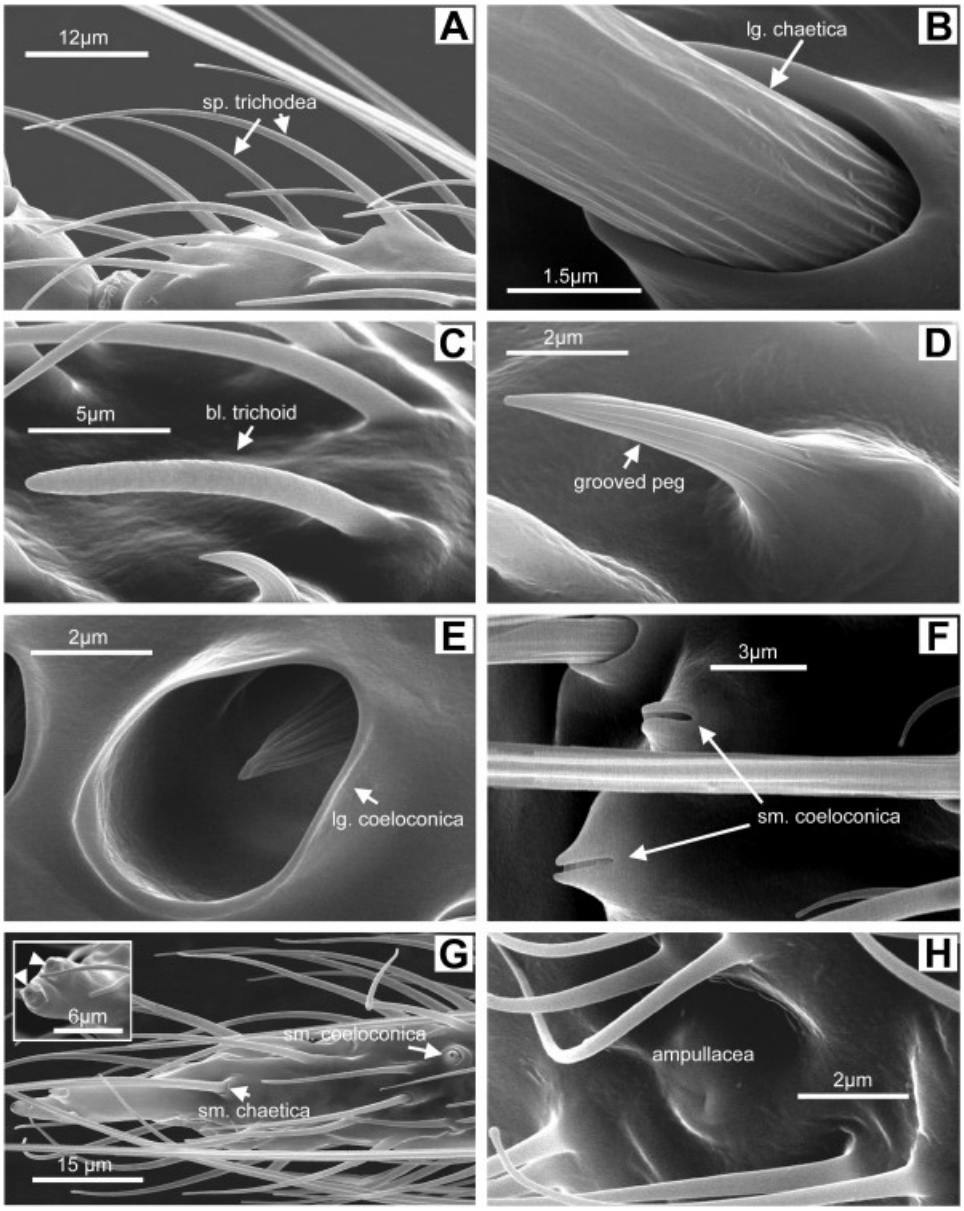


Figure 4. *An.gambiae* antennal sensilla (Pitts and Zwiebel 2006). A: Sensilla trichodea (sharp). B: Socket of large chaetica sensillum. C:Sensilla trichodea (blunt). D: Basiconic sensillum (grooveg peg). E: Large coeloconic sensillum. F: Small coeloconic sensilla. G: Small chaetica and small coelocoinc sensilla (inset shows small coeloconic sensilla at distal tip of antennae). H: Sensillum ampulacea.

The most numerous sensilla found along the *An. gambiae* antennae are the sensilla trichodea (or trichoid), which comprise two-thirds of all sensilla (Pitts and Zwiebel 2006). More than 600 trichoid sensilla are located on each *An. gambiae* female antenna (Pitts and Zwiebel 2006), while only about 250 are found on each male antenna (McIver 1982). Some mosquitoes have as many as 5 distinct sub-classes of sensilla trichodea based on their lengths, shapes, and wall thicknesses (Boo 1980). Two or three distinct types of sensilla trichodea are recognized in *An. gambiae* (Pitts and Zwiebel 2006; Qiu et al. 2006; Meijerink and van Loon 1998). The sharp trichodea vary in length but all taper noticeably from base to tip, have a smooth surface without obvious grooves or ridges, and protrude directly from the cuticle surface. Blunt trichodea are distinct in that they do not taper sharply, ending instead in a rounded tip that is nearly as wide as the base. These sensilla are more uniform in length than the sharp trichodea. In *An. gambiae*, blunt sensilla trichodea are are found in small numbers on with females having 15–20 per antenna (Pitts and Zwiebel 2006). Almost all sensilla trichodea house 2 ORNs (McIver 1982). Single sensillum reocrdings from sensilla trichodea in *An. gambiae* have shown that the ORNs are sensitive to a range of carboxylic acids, alcohols, ketones, and ammonia (Qiu et al. 2006; Meijerink et al. 2001; Meijerink and van Loon 1999; van den Broek and den Otter 1999). At least 6 different physiological classes of trichoid sensilla have been named based on odor response profiles (Qiu et al. 2006). Importantly, some of the

specific odors tested: geranyl acetone, 3-methyl-1-butanol, 6-methyl-5-heptene-2-one, and indole, are known components of human sweat (Cork and Park 1996; Qiu et al. 2006). Finally, while many ORNs exhibit increases in action potentials (excitation) when exposed to odors, some ORNs exhibit decreases in spontaneous action potentials (inhibition). Often the same odor can elicit excitatory responses in one neuron, but inhibitory responses in another (Qiu et al. 2006; Meijerink et al. 2001; Meijerink and van Loon 1999; van den Broek and den Otter 1999). Likewise a given neuron may be excited by one odor but inhibited by another. It is very likely that the combination of excitation of groups of ORNs and the inhibition of others is what ultimately determines how the mosquito perceives an odor. Evidence for this type of odor coding in insects comes from studies showing differential patterns of neuronal activity in the brain antennal lobes upon stimulation of chemosensory appendages with different odors (Masse et al. 2009; Riffel et al. 2009). Antennal lobes are the sites of first order connections made by the ORNs as they project into the brain from the antennae, via the antennal nerve. The structures of mosquito antennal lobes have been described and are similar to those of other insects, such that odor coding is likely to be processed similarly (Ghaninia et al. 2007; Ignell et al. 2005; Anton et al. 2004, 2003).

The sensilla basiconica, or grooved pegs are the second most numerous class of antennal sensilla and closely resemble the sensilla

basiconica of *Aedes aegypti* and other Culicines (McIver 1982). Externally they resemble thorns having 10–12 grooves along their surfaces and are raised on small cuticular prominences. Grooved pegs are found in small numbers on the basal flagellomeres and increase in number distally, with nearly half of them occurring on the last three flagellomeres (Pitts and Zwiebel 2006). Subclasses of grooved pegs have been distinguished in other Anophelines, based on numbers of external grooves, wall structures, and numbers of innervating neurons (Boo and McIver 1976), but not in *An. gambiae*. Each peg houses 2-4 olfactory neurons (McIver 1982) and is sensitive to important volatile chemicals. ORNs in grooved pegs of *Ae. aegypti* were the first to be recognized in mosquitoes as being sensitive to lactic acid, a major component of human sweat (Davis and Sokolove 1976). Grooved peg nerons in *An. gambiae* are also sensitive to lactic acid as well as other important kairomones like ammonia and butylamine. *An. gambiae* grooved pegs have been grouped into 5 physiological types (GP1-5) based on their response profiles to a common set of odorants (Qiu et al. 2006). All grooved pegs respond with excitation to ammonia and butylamine (Qiu et al. 2006). Four of the five types respond to a few carboxylic acids, some by excitation some by inhibition (Qiu et al. 2006). On the whole grooved peg neurons seem to be more specialized with respect to the breadth of odor responses than trichoid sensilla neurons, at least for the panels of odors that have been used in published studies.

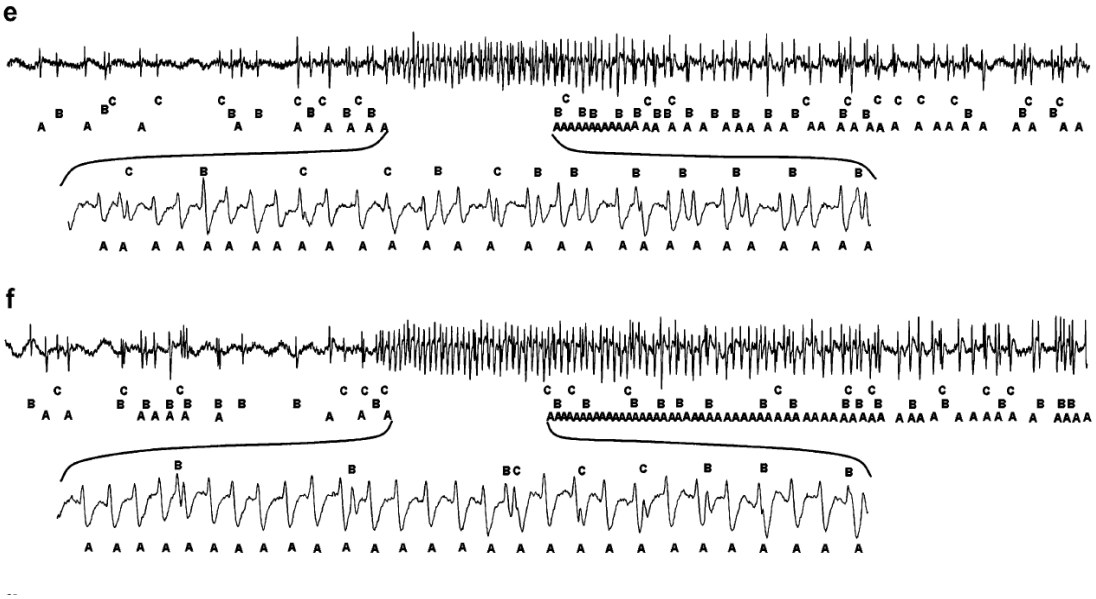


Figure 5. Response profiles of *An. gambiae* grooved peg sensilla to ammonia (top panel) and lactic acid (bottom panel, Qiu et al. 2006). Clear increases in action potential frequencies (vertical spikes) are induced by odor application. Amplitudes of spikes are characteristic of a specific neuron, here named A, B, or C. In both cases the A neuron responds to odor. For other odors, the B or C neurons may respond (not shown).

The secondary chemosensory organs on *An. gambiae* heads are the maxillary palps and proboscis. The palps are located latero-ventrally from the antennae and are comprised of five segments in both sexes. The palps of females are longer than the antennae in Anophelines, but not in Culicines (McIver 1982), which can be used as a defining characteristic in subfamily identification. The distal 2 segments of male *An. gambiae* maxillary palps are fused and form a club-shaped end. The purpose of this club is unknown. The palps of mosquitoes are also distinguished by the presence of a single morphological sensillar type, the capitate peg (Sutcliffe 1994; McIver 1982). Contained within the capitate pegs are 3 ORNs, one of which has a specialized folded (lamellate) structure (McIver 1982). This neuron is sensitive to increasing concentrations of CO_2 in many insects including the mosquitoes *Ae. aegypti* and *An. gambiae* (Lu et al. 2007; Grant and O'Connell, 1996). The two other ORNs in capitate pegs of *An. gambiae* are excited volatile odors. One of them is a highly sensitive to 1-octen-3-ol that is both a human sweat component and a plant volatile (Lu et al. 2007). The remaining ORN is sensitive to a range of compounds, but is most highly excited by 2,4,5 trimethyl thiazole (Lu et al. 2007).

The mosquito proboscis is a modified labium and is the most ventral of the head appendages, ending in paired labellar lobes. The proboscis houses the stylets that are used by both sexes for nectar feeding and are also used by females for bloodfeeding (Clements 1992).

Until recently, the labellum was considered to be solely gustatory in its function (McIver 1982), but is now recognized as also having an olfactory capacity (Kwon et al. 2006; Melo et al 2004). Specifically, small but numerous T2 sensilla located on both labellar lobes each house 2 ORNs that are sensitive to volatile odors, including the important kairomones butylamine and oxovaleric acid (Kwon et al. 2006). The future exploration of the role of the proboscis in host seeking and selection will be interesting to follow.

Molecular Basis of Chemosensation

The first insect odorant receptors (*Ors*) were identified in *D. melanogaster* by multiple groups using differing approaches (Clyne et al. 1999; Gao and Chess 1999; Vosshall et al. 1999). As a result of genome sequencing projects, *Or* gene families have now been described in numerous insect species representing multiple orders (Smith et al. 2011; Robertson et al. 2010; Smadja et al. 2009; Robertson and Wanner 2006; Krieger et al. 2003; Robertson et al. 2003), including three mosquito species: *An. gambiae* (Hill et al. 2002), *Ae. aegypti* (Bohbot et al. 2007), and *Culex quinquefasciatus* (Pelletier et al. 2010). Insect *Ors* are an extremely divergent gene superfamily, often sharing very low amino acid identities/similarities within the same species, and having few orthologs between species (Robertson et al. 2003; Robertson 2006; Ache and Young 2005). They represent a distinct subclass (class E) of the very

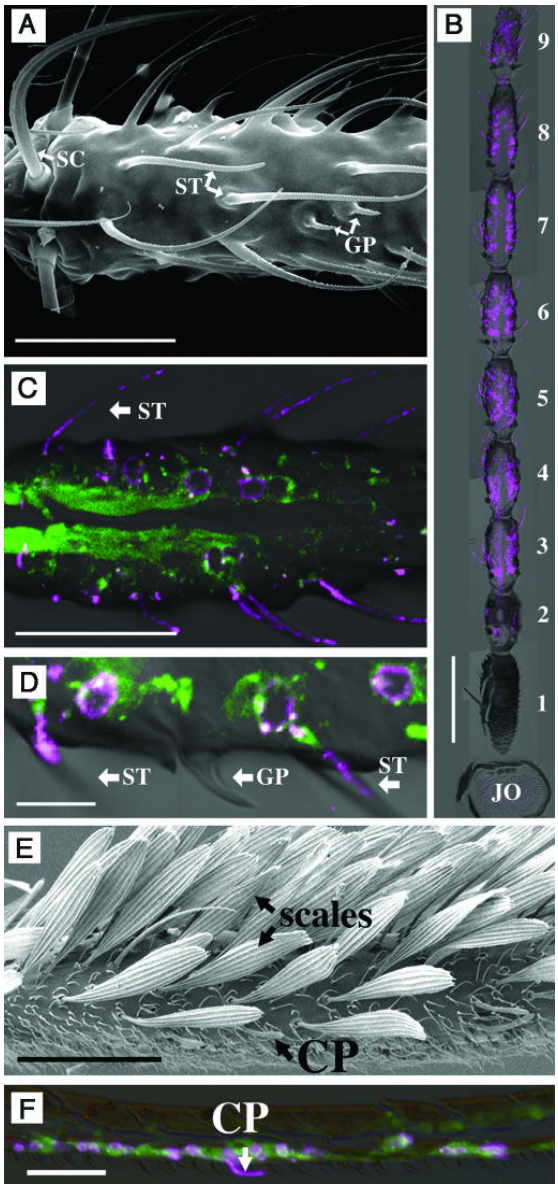
large G protein-coupled receptor (GPCR) family with a unique evolutionary history and no homologs in vertebrate genomes (Kaupp 2010; Ramdya and Benton 2010; Robertson et al. 2003). *Ors* are usually widely dispersed in insect genomes, with some tandem or clustered groups of paralogous genes representing recent duplications. Moreover, single nucleotide polymorphisms are widespread, very few pseudogenes have been retained, and numerous duplications are evident (Hill et al. 2002, Robertson et al. 2003; Ache and Young 2005; Bohbot et al. 2007), all suggesting that insect odorant receptors are very rapidly evolving genes (Sánchez-Gracia et al. 2009).

The large family of 79 candidate *An. gambiae Or* genes (*AgOr*) was identified using homology-based approaches (Fox et al., 2001, Fox et al. 2002; Hill et al 2002). A few *AgOrs* are clustered in the genome and, based on their homologies, similar directions of transcription, and conserved introns, are likely to have arisen as a result of multiple duplication events (Hill et al., 2002). In general the conceptual translations of *AgOrs* do share the common topological feature of 7 predicted transmembrane (TM) domains, although fewer than 7 TMs are predicted for some (Hill et al. 2002). Like other insect *Ors*, the *AgOr* gene products share little identity at the primary amino acid level, usually less than 20%, although 14 pairs share >70% peptide identity (Hill et al. 2002). Phylogenetic comparison of the *DmOrs* and *AgOrs* best illustrates the divergence between these insect odorant receptors as nearly all clades

are composed of within-species receptors, with a few clades being composed of interspecies groupings (Hill et al., 2002).

As expected for chemosensory receptors, insect *Ors* are expressed in adult antennae, maxillary palps (Figure 6) as well as larval chemosensory structures (Clyne et al., 1999, Gao and Chess, 1999, Vosshall et al., 1999, Vosshall et al., 2000; Fox et al. 2001; Fox et al. 2002; Hill et al. 2002; Melo et al. 2004; Xia and Zwiebel 2006; Bohbot et al. 2007; Pelletier et al. 2010; Krieger et al. 2003). Their expression is limited to specific sensillar types in stereotypic regions of the antennae and their patterns and projections are conserved from individual to individual (Schymura et al. 2010; Sakurai et al. 2004; Vosshall et al., 2000). One *Or* gene is highly conserved across insect orders, is widely co-expressed with other *Ors*, and is required for their function (Krieger et al. 2003; Pitts et al. 2004; Larsson et al. 2004; Jones et al. 2005; Xia and Zwiebel 2006). A more detailed discussion of this receptor is provided below.

The regulation of *Or* expression has been explored in *D. melanogaster* where two genes, *acj6* and *pdm3*, encode POU-domain transcription factors that are expressed in ORNs and modulate expression of several *DmOrs* (Bai et al. 2009; Tichy et al. 2008). *Or* expression patterning is likely to play a significant role in establishing olfactory sensitivity, indeed *acj6* mutants show defects in olfactory responses (Clyne et al. 1999). Cis-acting DNA elements that are involved in *Or*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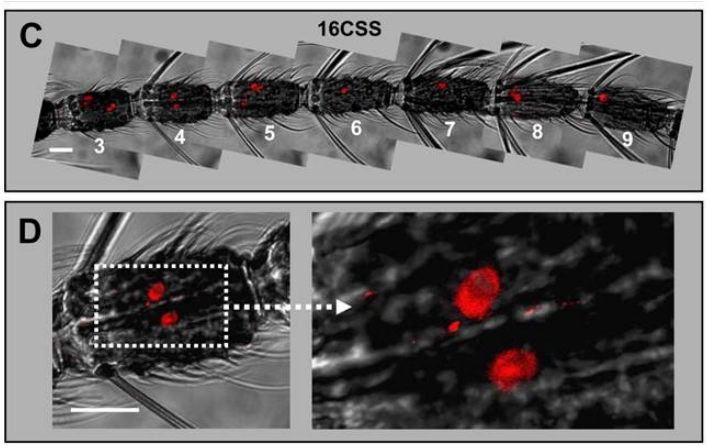
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Figure 6. *AgOr* expression in *An. gambiae* female chemosensory appendages. **Top Panel** - A: scanning electron micrograph of a single flagellomere showing sensilla types. B: Antibodies against AgOR7 protein reveal its broad expression (magenta) in female antennae. C: AgOR7 is localized to cell bodies and dendrites of sensilla trichodea (ST) neurons (labeled green) D: AgOR7 is absent in grooved peg (GP) neurons. E: scanning electron micrograph of a maxillary showing single sensilla type. F: AgOR7 is localized to cell bodies and dendrites of capitate peg sensilla (CP) neurons (green; Pitts et al. 2004). **Bottom panel** – C: in situ probes detect *AgOr1* in a few cells in each antennal flagellomere. D: Higher magnifications of the 4th flagellomere from C (Schymura et al 2010).

transcriptional regulation, both positively and negatively, have also been characterized in *D. melanogaster* (Ray et al. 2007, 2008). Whether those elements are conserved in *An. gambiae* remains to be determined. However, strong homologs of both *acj6* and *pdm3* are expressed at high levels in *An. gambiae* antennae (unpublished observation). Furthermore expression of *AgOrs* in the *An. gambiae* antennae is limited to the trichoid sensilla (Pitts et al. 2004). This is analogous to the expression pattern observed for *DmOrs* (Vosshall et al. 2000). Therefore it is likely that the odor sensitivities of *An. gambiae* trichoid sensilla that are described above can be accounted for by the repertoire of *AgOrs* that are localized in trichoid ORNs. Still unknown, as of the time of this writing, was the identity of chemoreceptors expressed in grooved peg neurons in *An. gambiae*. The discovery of a new class of chemoreceptors in *D. melanogaster* that function in odor sensing in the morphological analogs of grooved pegs (Benton et al. 2009) led directly to the identification of a new receptor class in *An. gambiae* that may underlie odor sensitivities in this important class of sensilla. Chapter II will describe this work in detail.

Ors from multiple insect species have now been functionally characterized (for review see Kaupp 2010; Ramdya and Benton 2010; and Touhara and Vosshall 2009). Nearly the entire repertoires of *Ors* in *D. melanogaster* (Hallem et al. 2004a) and *An. gambiae* have been deorphaned (Carey et al. 2010; Wang et al. 2010). The receptors can be divided into 2 broad classes based upon specificity of function: the

pheromone receptors activated by blends of compounds that are released by conspecifics, and the general odorant receptors activated or inhibited by a range of semiochemicals that are released by heterospecifics (Touhara and Vosshall 2009). Based on heterologous expression studies, *AgOrs* seem to belong to the latter class as no true pheromonal functions have been described for them, although a few receptors are sensitive to a narrow range of odors and odor ligands are unknown for several more (Hallem et al. 2004b; Xia et al. 2008; Bohbot et al. 2010; Carey et al. 2010; Wang et al. 2010). Perhaps future studies will identify important *An. gambiae* pheromone(s) and the receptor(s) that mediate their sensitivities.

In heterologous expression studies *AgOrs* were found to be sensitive to chemical classes such as heterocyclics, aromatics and alcohols (Carey et al 2010; Wang et al. 2010). Among these compounds are odors found in human sweat (Carey et al. 20101; Wang et al. 2010). However, receptors for amines, including ammonia, were clearly lacking (Carey et al 2010; Wang et al. 2010). This again suggests that chemoreceptors other than *AgOrs* are responsible for ammonia sensitivity in grooved pegs. In another study *AgOr1* conferred sensitivity to 2-methylphenol (Hallem et al. 2004b), a component of human sweat that elicits responses in mosquito antennae (Cork and Park 1996). Interestingly, *AgOr1* is also down-regulated in female antennae after blood-feeding (Fox et al. 2001). Whether or not *AgOr1* is required for 2-methylphenol sensitivity in a living, adult mosquito awaits validation. If

demonstrated, such a finding would close a significant gap in our understanding of mosquito olfaction. Only one study to date has demonstrated the functional requirements of *AgOrs* in living animals by using RNA interference (RNAi) knockdowns of gene expression. This study will be discussed in Chapter II, where knockdowns of different classes of receptors in *An. gambiae* larvae led to altered behavioral responses to distinct odors thus defining distinct signaling pathways (Liu et al. 2010).

Gustatory (i.e. taste) receptors (*Grs*) have been identified in numerous insect genomes (Robertson et al. 2010; Kent and Robertson 2009; Robertson and Kent 2009; Kent et al. 2008; Robertson and Wanner 2006). These large gene families appear to encode 7 transmembrane receptor proteins and are structurally and phylogenetically similar to the *Ors* (Robertson et al. 2003). *Grs* have been studied most extensively in *D. melanogaster* where they are responsible for sensing general classes of tastants like sweet, bitter, and carbonation (Weiss et al. 2011; Dahanakar et al. 2007; Jones et al. 2007; Kwon et al. 2007; Slone et al. 2007; Thorne et al. 2005). Perhaps most important to mosquito host-seeking are the carbon dioxide (CO_2) sensitive receptors (Lu et al. 2007). CO_2 is a powerful attractant to many bloodsucking insects and is especially important as an activator of mosquito flight (Lacey and Cardé 2010; Dekker et al. 2005; Gillies 1980). The *An. gambiae* homologs of CO_2 receptors are expressed in a specialized neuron in each capitate peg of

the maxillary palps and mediate the highly sensitive phasic-tonic response to changes in carbon dioxide (Lu et al. 2007).

Odorants, which are often lipophilic, must transverse the sensillum lymph in order to activate odorant receptors. Families of odorant binding proteins (*Obps*) are thought to fulfill this role in insect sensilla (Bloomquist and Vogt 2003). *Obps* were first discovered in the silk moth *Antheraea polyphemus* where a pheromone-binding protein (*Apol*PBP) was shown to interact with a female sex pheromone component in the lymph of a pheromone sensitive sensillum (Vogt and Riddiford 1981). Members of this gene family share some common characteristics: N-terminal signal sequence, putative odor binding capability, overall small size of approximately 14kDa, and the presence of alpha helices each containing conserved cysteine residues at characteristic positions, but are otherwise extremely divergent (Bloomquist and Vogt 2003). The identification of *Obps* in several insect species (Xu et al. 2003; Nikonov et al. 2002; Briand et al. 2001; Kim et al. 1998; Picone et al. 2001; Riviere et al. 2003; Vogt et al. 1999) and the description of a new subfamily of *Obps* called General Odorant-Binding Proteins (*Gobps*) in Lepidoptera provided evidence that *Obps* are part of multigene families with intrinsic roles in peri-reception (Vogt et al., 1989, Vogt et al., 1991). The availability of the *D. melanogaster* and *An. gambiae* genomes resulted in the identification of *Obp* families in both species (Hekmat-Scafe et al. 2002; Xu et al. 2003).

In *An. gambiae*, 57 *Obp* genes were identified in homology-based searches (Xu et al. 2003). Like the *AgOrs*, *AgObps* show very little sequence homology either within or across species (Xu et al. 2003). *AgObps* are clustered in the *An. gambiae* genome and belong to three subclasses. The 29 classical *Obps* are predicted to form a six-alpha helical structure held together by the interactions of 6 conserved cysteine residues, the 12 Plus-C *Obps* contain an additional exon/intron and other conserved amino acid residues that are not found in the classical *Obps*, and the 16 atypical *Obps* encode peptides with longer C-terminal regions that contain additional conserved cysteine residues (Xu et al 2003). Atypical *AgObps* lack introns, but are otherwise more similar to the classical *AgObp* genes than the Plus-C *AgObps.* The atypical *AgObps* are not found in *D. melanogaster* leading to the speculation that this subfamily arose after fly/mosquito divergence (Xu et al. 2003).

The first evidence for *Obp* olfactory function in Dipteran flies was provided by the characterization of the *Drosophila OBP76a* mutant, also called *Lush*. *Lush* mutants display abnormal attraction to toxic levels of ethanol (Kim et al., 1998) and loss of sensitivity to the aggregation pheromone 11-cis vaccinyl acetate (VA) (Xu et al. 2005). These 2 compounds are detected by two different subsets of trichoid sensilla each expressing *Lush*. Transgenic rescue and the introduction of *Lush* protein into the recording pipette of VA sensitive neurons in mutant flies restored the electrophysiological response to this compound. However, introducing

an alternative *Obp*, *83a*, failed to restore the VA response, demonstrating that *Lush* is necessary for the transduction pathway of VA (Xu et al. 2005). Moreover, modification of *Lush* protein by making single amino acid substitution mimics the conformation change associated with VA binding and induces activation of the pheromone receptor (Laughlin et al. 2008).

If high *Obp* expression in chemosensory tissues implies olfactory function then expression of *Obps* in non-chemosensory tissues either indicates cryptic chemosensory cells in thode tissues, or alternative functions for *Obps*. In *D. melanogaster* (Galindo and Smith, 2001) the *Obps* have been further classified based upon their expression pattern: olfactory, taste, olfactory & taste and non-chemosensory. The non-chemosensory class includes the *Drosophila Obp19d* (*pbprp2*) whose expression is ubiquitous (Park et al. 2000; Shanbhag et al. 2001; Galindo and Smith 2001) and is expressed in various olfactory and taste organs and in the epidermis. Accordingly, an odorant scavenger function has been suggested for these *Obps* (Shanbhag et al. 2001; Park et al. 2000). In *An. gambiae*, functional roles for *AgObps* in olfactory signaling have been described recently. RNA intereference knockdown of *AgObp1* expression reduced the antennal response to indole (Biessmann et al. 2010). Another study, using recombinant proteins in vitro, showed that ligand binding properties were altered when combinations of *AgOBPs* were mixed together (Qiao et al 2010). The study also showed co-expression of *AgObps* in the same antennal sensilla. The authors

conclude that *AgObps* may form heterodimers that work cooperatively to bind odors, which would effectively increase the odor receptive range (Qiao et al, 2010). These studies have offered new insights into the functional properties of *AgObps*. The demonstration that RNAi-mediated knockdown of *AgOrs* (Chapter II) and *AgObps* (Biessmann et al. 2010) can produce deficits in odor sensitivities should encourage similar studies designed to elucidate not only their independent roles, but perhaps co-dependence of these two gene classes. A detailed description of the expression pattern of the *AgObps* family is found in Chapter III. Moreover, the identification of genes that are similar to families of lipophilic carriers and highly expressed in chemosensory tissues, but are unrelated to the *AgObps*, suggests novel odorant binding functions for them (Chapter III).

Olfactory Signal Transduction

From its first identification and subsequent expression analysis, *DmOr83b* was viewed as a potentially extraordinary odorant receptor (Vosshall et al., 2000). It is longer than the average DmOR, having an extended loop between the 4th and 5th putative transmembrane regions and it is expressed in nearly all antennae and maxillary palp neurons in *D. melanogaster* (Vosshall et al., 2000). Furthermore, *DmOr83b* and other *DmOrs* have unexpectedly inverted conformations in the dendritic membrane, with their N-termini located intra-cellularly and their C-termini located extra-cellularly (Benton et al., 2006; Lundin et al. 2007; Tsitoura et

al. 2010). Moreover, highly conserved orthologs have been identified in all insect genomes sequenced to date, some of which have been used to complement the general anosmia in *DmOr83b*$^{-}$ null mutant flies (Larsson et al. 2004; Neuhaus et al. 2005; Jones et al. 2005). *DmOr83b* was also demonstrated to be necessary and sufficient for the proper localization and retention of other conventional *DmOrs* to the dendritic membrane (Larsson et al., 2004, Benton et al., 2006). The *DmOr83b* subgroup is unique to insects and forms a monophyletic clade within the larger insect chemoreceptor family and bridges the *Ors* to gustatory receptors (Robertson et al., 2003). This exceptional degree of sequence conservation and expression characteristics among insect OR gene families suggested that the *DmOr83b* family represents a non-conventional *Or* that is required for olfactory signal transduction in all insects.

Heterologous expression studies revealed that orthologs of *DmOr83b* partner with conventional ORs and enhance odorant response sensitivities (Wetzel et al. 2001; Sakurai et al. 2004; Neuhaus et al., 2005). *DmOr83b* does not independently confer odorant sensitivity (Dobritsa et al. 2003; Benton et al. 2006; Sato et al. 2008; Wicher et al. 2008), but forms an ion channel either alone or in cooperation with a conventional *Or* that is gated by its cognate odor ligand (Sato et al. 2008; Wicher et al. 2008; Smart et al. 2008). Still controversial is whether the heteromeric Or83b/Orx receptor can also function as a metabotropic receptor, coupled

to G-protein second messenger signaling (Nakagawa and Vosshall 2009; Sato and Touhara 2009). The molecular machinery and mechanisms of insect olfactory signal transduction is thus distinct from vertebrate and nematode signaling, each of which utilize GPCR signaling (Figure 7). As the downstream signaling events in insect chemoreception are very poorly understood they will not be covered in this introduction. The reader is directed to the following reviews for further reading (Kaupp 2010; Nakagawa and Vosshall 2009; Rutzler and Zwiebel 2005). It will now be interesting to study the evolutionary history of this signaling pathway. We may indeed find that the principles of insect olfaction are conserved in more basal hexapod or arthropod lineages, and potentially providing opportunities for broad-based control strategies that rely on olfaction for their implementation.

Preview of Chapters II, III, and IV

What follows are accounts of studies that I have personally contributed to that were designed to answer questions about the molecular basis of mosquito olfaction. As already mentioned, Chapter II will describe the identification of a new class of candidate chemoreceptors in *An. gambiae*, the *ionotropic receptors*. This large family of genes is weakly homologous to a family first discovered in olfactory tissues of *D. melanogaster*, the *DmIrs* (Benton et al. 2009). The *Irs* of both species are related to the ionotropic glutamate receptors and have apparently evolved

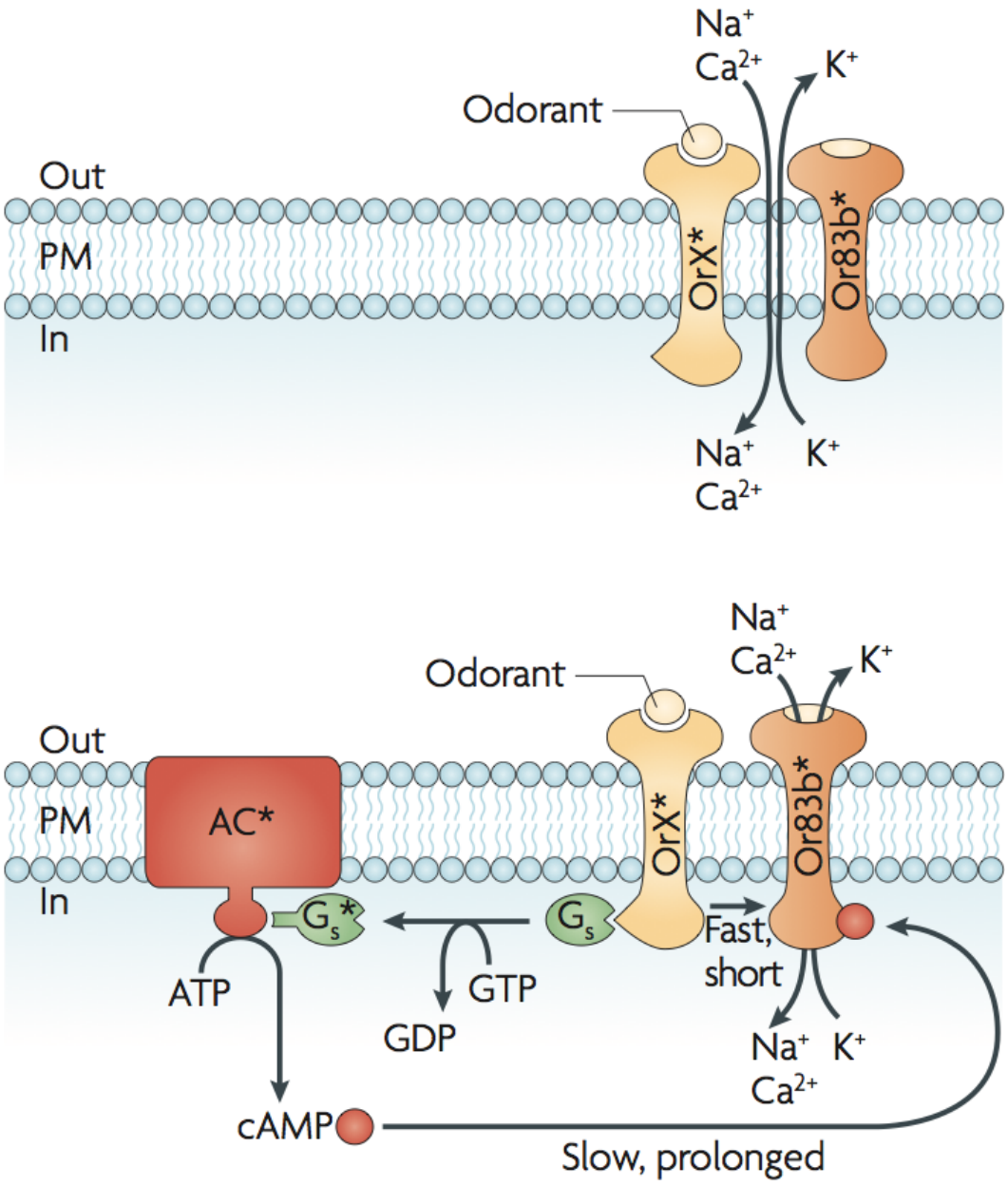


Figure 7. Model of insect olfactory signal transduction (Kaupp 2010). Or83b (or its ortholog in other species) forms a channel either in cooperation with another OrX (top panel) or alone (bottom panel). Whether some OrX-Or83b complexes can also signal via G proteins (bottom panel) remains controversial.

as sensory receptors. Their expression is significantly enhanced in *An. gambiae* antennae and maxillary palps, and in *D. melanogaster* they are responsible for odor sensitivity in sensilla that are the morphological analogs of mosquito grooved pegs. We demonstrate a requirement for one of these receptors, *AgIr76b*, in larval behavioral responses to butylamine. Furthermore, the butylamine response is insensitive to the knockdown of *AgOr7*, indicating that distinct olfactory signaling pathways exist in *An. gambiae* larvae.

In Chapter III, my coauthors and I examine transcriptome profiles of the antennae and maxillary palps of female and male *An. gambiae*. The studies were designed to explore several aspects of the molecular components of *An. gambiae* chemosensory tissues using RNA-seq technology. First, we wanted to look for genes that are enhanced in chemosensory tissues, with the goal of identifying new olfactory signaling players. These genes, if identified, could become targets for pharmacological screens aimed at developing novel mosquito repellents or attractants. Second, we were interested in potential differences in gene expression between antenna and maxillary palps and between sexes. Finally, we could use the data to carefully study the expression profiles of *AgOrs*, *AgIrs*, *AgGrs*, and *AgObps*, the large chemosensory gene families. What we found was enlightening. We identified classes of genes that had previously not been associated with chemoreception, including potentially novel odorant binding proteins. Moreover, we discovered that the

complexity of chemoreceptor expression was similar in antennae and maxillary palps females and males, although their absolute expression levels were higher in females. These results could impact future studies of mosquito olfaction and we expect will provide a valuable resource for the research community.

Chapter IV fits loosely into the overall work and is the product of an ecological study of *An. gambiae* in Ghana in which I participated in the summer of 2010. I became friends with Drs. Derek Charlwood and Alexander Egyir-Yawson while attending mosquito conferences in July 2008 and January 2010. They invited me to come to Ghana to assist in their work. Through the generous gift of Dr. Gisela Mosig, who bequeathed money to the Department of Biological Sciences for graduate student travel, I was able to accept the invitation. The survey was designed to examine the bionomics of the local *An. gambiae* population including, breeding sites, molecular forms, age structure, survival rates, and gonotrophic cycle timing in a small rice growing village. The 2010 study was a follow-up to one conducted a year earlier. The ultimate goal of this work would be to understand the vectorial capacity of *An. gambiae* in the village such that appropriate mosquito control measures might be implemented.

References

Ache BW and Young JM (2005) Olfaction: Diverse species, conserved principles. *Neuron* 48: 417-30.

Ansell J, Hamilton KA, Pinder M, Walraven GE, and Lindsay SW (2002) Short-range attractiveness of pregnant women to *Anopheles gambiae* mosquitoes. *Trans R Soc Trop Med Hyg* 96: 113-6.

Anton S and Rospars JP (2004) Quantitative analysis of olfactory receptor neuron projections in the antennal lobe of the malaria mosquito, *Anopheles gambiae*. *J Comp Neurol* 475: 315-26.

Anton S, van Loon JJ, Meijerink J, Smid HM, Takken W, and Rospars JP (2003) Central projections of olfactory receptor neurons from single antennal and palpal sensilla in mosquitoes. *Arthropod Struct Dev* 32: 319-27.

Barat LM, Palmer N, Basu S, et al (2004) Do malaria control interventions reach the poor? A view through the equity lens. *Am J Trop Med Hyg* 71: S174–8.

Barr AR (1967) Occurrence and Distribution of the *Culex pipiens* Complex. *Bull Wld Hlth Org* 37: 293-296.

Bates I, Fenton C, Gruber J, et al. (2004) Vulnerability to malaria tuberculosis, an HIV/AIDS infection and disease. Part 1: Determinants operating at individual and household level. *Lancet Infect Dis* 4: 267–77.

Benton R, Vannice KS, Gomez-Diaz C, and Vosshall LB (2009) Variant ionotropic glutamate receptors as chemosensory receptors in Drosophila. *Cell* 136: 149-62.

Benton R, Sachse S, Michnick SW, Vosshall LB (2006) Atypical membrane topology and heteromeric function of Drosophila odorant receptors in vivo. *PLoS Biol* 4: e20.

Bernier UR, Kline DL, Barnard DR, Schreck CE, Yost RA (2000) Analysis of human skin emanations by gas chromatography/mass spectrometry. 2. Identification of volatile compounds that are candidate attractants for the yellow fever mosquito (*Aedes aegypti*). *Anal Chem* 72: 747–756.

Besansky NJ, Hill CA, and Costantini C (1994) No accounting for taste: host preference in malaria vectors. *Trends Parasitol* 20: 249-51.

Biessmann H, Andronopoulou E, Biessmann MR, Douris V, Dimitratos SD, Eliopoulos E, Guerin PM, et al. (2010) The *Anopheles gambiae* odorant binding protein 1 (AgamOBP1) mediates indole recognition in the antennae of female mosquitoes. *PLoS One* 5: e9471.

Blackwell A and Johnson SN (2000) Electrophysiological investigation of larval water and potential oviposition chemo-attractants for *Anopheles gambiae* s.s. *Ann Trop Med Parasitol* 94: 389-98.

Bloomquist GJ and Vogt RG, eds. (2003) **Insect Pheromone Biochemistry and Molecular Biology: The Biosynthesis and Detection of Pheromones and Plant Volatiles**. Elsevier.

Bock GR and Cardew G, eds. (1996) **Olfaction in Mosquito-Host Interactions.** John Wiley and Sons.

Bohbot JD, Fu L, LE TC, Chauhan KR, Cantrell CL, and Dickens JC (2011) Multiple activities of insect repellents on odorant receptors in mosquitoes. *Med Vet Entomol* Mar 14. [Epub ahead of Print]

Bohbot JD, Jones PL, Wang G, Pitts RJ, Pask GM, and Zwiebel LJ (2010a) Conservation ofindole responsive odorant receptors in mosquitoes reveals an ancient olfactory trait. *Chem Senses* 36: 149-60.

Bohbot JD and Dickens JC (2010b) Insect repellents: modulators of mosquito odorant receptor activity. *PLoS One* 5: e12138.

Bohbot JD and Dickens JC (2009) Characterization of an enantioselective odorantreceptor in the yellow fever mosquito *Aedes aegypti*. *PLoS One* 4: e7032.

Bohbot J, Pitts RJ, Kwon HW, Rutzler M, Robertson HM, and Zwiebel LJ (2007) Molecular characterization of the *Aedes aegypti* odorant receptor gene family. *Insect Mol Biol* 16:525-37.

Boo KS (1980) Fine structure of the antennal sensory hairs in female *Anopheles stephensi*. *Z Parasitenkd (Parasitol Res)* 61: 161-71.

Boo KS, McIver SB (1976) Fine structure of surface and sunken grooved pegs on the antenna of female *Anopheles stephensi* (Diptera: Culicidae). *Can J Zool* 54: 235-44.

Briand L, Nespoulous C, Huet JC, Takahashi M, and Pernollet JC (2001) Ligand binding and physico-chemical properties of *asp2*, a recombinant odorant-binding protein from honeybee (*Apis mellifera* l.). *Eur J Biochem* 268**:** 752-60.

Byrne JP (2008) **Encyclopedia of Pestilence, Pandemics, and Plagues: A-M.** Greenwood.

Carey AF, Wang G, Su CY, Zwiebel LJ, and Carlson JR (2010) Odorant reception in the malaria mosquito *Anopheles gambiae*. *Nature* 464: 66-71.

Charlwood JD, Tomás EV, Salgueiro P, Egyir-Yawson A, Pitts RJ, and Pinto J (2011) Studies on the behaviour of peridomestic and endophagic M form *Anopheles gambiae* from a rice growing area of Ghana. *Bull Entomol Res* Mar 15:1-7. [Epub ahead of print]

Charlwood JD and Jones MDR (1980) Mating in the mosquito, *Anopheles gambiae* s.l. 2. Swarming behavior. *Physiol Entomol* 5: 315-20.

Charlwood JD and Jones MDR (1979) Mating-Behavior in the mosquito, *Anopheles gambiae* s.l. 1. Close range and contact behavior. *Physiol Entomol* 4: 111-20.

Choi HW, Breman JG, Teutsch SM, et al (1995) The effectiveness of insecticide impregnated bednets in reducing cases of malaria infection: A meta-analysis of published results. *Am J Trop Med Hyg* 52: 377–82.

Clements AN (1992) **The Biology of Mosquitoes. Volume 1**. Chapman and Hall.

Clements AN (1999) **The Biology of Mosquitoes. Volume 2**. Chapman and Hall.

Clyne PJ, Warr CG, Freeman MR, Lessing D, Kim J, and Carlson JR (1999) A novel family of divergent seven-transmembrane proteins: candidate odorant receptors in Drosophila. *Neuron* 22: 327-38.

Cook SM, Khan ZR, and Pickett JA (2007) The use of push-pull strategies in integrated pest management. *Annu Rev Entomol* 52: 375-400.

Cork A and Park KC. Identification of electrophysiologically-active compounds for the malaria mosquito, *Anopheles gambiae*, in human sweat extracts. *Med Vet Entomol* 10: 269-76.

Costantini C, Birkett MA, Gibson G, Ziesmann J, Sagnon NF, Mohammed HA, Coluzzi M, and Pickett JA (2001a) Electroantennogram and behavioural responses of the malaria vector *Anopheles gambiae* to human-specific sweat components. *Med Vet Entomol* 15: 259-66.

Costantini C and Diallo M (2001b) Preliminary lack of evidence for simian odour preferences of savanna populations of *Anopheles gambiae* and other malaria vectors. *Parassitologia* 43: 179-82.

Costantini C, Sagnon N, della Torre A, Coluzzi M (1999) Mosquito behavioural aspects of vector-human interactions in the *Anopheles gambiae* complex. *Parassitologia* 41: 209-17.

Costantini C, Sagnon NF, della Torre A, Diallo M, Brady J, Gibson G, and Coluzzi M (1998) Odor-mediated host preferences of West African mosquitoes, with particular reference to malaria vectors. *Am J Trop Med Hyg* 58: 56–63.

Costantini C, Gibson G, Sagnon N, Della Torre A, Brady J, and Coluzzi M (1996) Mosquito responses to carbon dioxide in a west African Sudan savanna village. *Med Vet Entomol* 10: 220-7.

Curtis C, Maxwell C, Lemnge M, et al (2003) Scaling-up coverage with insecticide-treated nets against malaria in Africa: Who should pay? *Lancet Infect Dis* 3: 304–7.

Dahanukar A, Lei YT, Kwon JY, and Carlson JR (2007) Two Gr genes underlie sugar reception in Drosophila. *Neuron*. 56: 503-16.

Davis EE and Sokolove PG (1976) Lactic acid-sensitive receptors on the antennae of the mosquito, *Aedes aegypti*. *J Comp Physiol* 105: 43-54.

Dekker T, Geier M, and Cardé RT (2005) Carbon dioxide instantly sensitizes female yellow fever mosquitoes to human skin odours. *Journal of Experimental Biology* 208: 2963-2972.

Dekker T, Takken W, and Braks MAH (2001) Innate preference for host-odor blends modulates degree of anthropophagy of *Anopheles gambiae* sensu lato (Diptera: Culicidae). *J Med Entomol* 38: 868-871.

Dekker T and Takken W (1998) Differential responses of mosquito sibling species *Anopheles arabiensis* and *An. quadriannulatus* to carbon dioxide, a man or a calf. *Med Vet Entomol* 12: 136-40.

Diatta M, Spiegel A, Lochouarn L, and Fontenille D (1998) Similar feeding preferences of *Anopheles gambiae* and *A. arabiensis* in Senegal. *Trans R Soc Trop Med Hyg* 92: 270–2.

Dobritsa AA, van der Goes van Naters W, Warr CG, Steinbrecht RA and Carlson JR (2003) Integrating the molecular and cellular basis of odor coding in the Drosophila antenna. *Neuron* 37: 827-41.

Duchemin JB, Tsy JM, Rabarison P, Roux J, Coluzzi M, and Costantini C, (2001) Zoophily of *Anopheles arabiensis* and *An. gambiae* in Madagascar demonstrated by odour-baited entry traps. *Med Vet Entomol* 15: 50–57.

Engsontia P, Sanderson AP, Cobb M, Walden KK, Robertson HM, and Brown S (2008) The red flour beetle's large nose: an expanded odorant receptor gene family in *Tribolium castaneum. Insect Biochem Mol Biol* 38: 387-97.

Fox AN, Pitts RJ, Robertson HM, Carlson JR and Zwiebel LJ (2001) Candidate odorant receptors from the malaria vector mosquito *Anopheles gambiae* and evidence of down-regulation in response to blood feeding. *Proc Natl Acad Sci U S A* 98**:** 14693-7.

Fox AN, Pitts RJ and Zwiebel LJ (2002) A cluster of candidate odorant receptors from the malaria vector mosquito, *Anopheles gambia*e. *Chem Senses* 27**:** 453-9.

Galindo K and Smith DP (2001) A large family of divergent Drosophila odorant-binding proteins expressed in gustatory and olfactory sensilla. *Genetics* 159: 1059-72.

Gao Q and Chess A (1999) Identification of candidate Drosophila olfactory receptors from genomic DNA sequence. *Genomics* 60: 31-9.

Ghaninia M, Hansson BS, and Ignell R (2007) The antennal lobe of the African malaria mosquito, *Anopheles gambiae* - innervation and three-dimensional reconstruction. *Arthropod Struct Dev* 36: 23-39.

Govella NJ, Chaki PP, Geissbuhler Y, Kannady K, Okumu F, Charlwood JD, Anderson RA, Killeen GF (2009) A new tent trap for sampling exophagic and endophagic members of the *Anopheles gambiae* complex. *Malaria J* 8:157.

Guerrant RL, Walker DH, and Weller PF, eds. (2006) **Tropical Infectious Diseases: Principles, Pathogens and Practice - Expert Consult: 2nd Edition.** Churchill Livingstone.

Grant AJ, Wigton BE, Aghajanian JG, and O'Connell RJ (1995) Electrophysiological responses of receptor neurons in mosquito maxillary palp sensilla to carbon dioxide. *J Comp Physiol A* 177: 389-96.

Hallem EA, Nicole Fox A, Zwiebel LJ, and Carlson JR (2004) Olfaction: mosquito receptor for human-sweat odorant. *Nature* 427: 212-3.

Hallem E, Ho MG, and Carlson JR (2004b) The molecular basis of odor coding in the Drosophila antenna. *Cell* 117: 965–979.

Hay SI, Sinka ME, Okara RM, Kabaria CW, Mbithi PM, Tago CC, Benz D, Gething PW, et al. (2010) Developing global maps of the dominant Anopheles vectors of human malaria. *PLoS Med* 7: e1000209.

Healy TP and Copland MJW (2001) Human sweat and 2-oxopentanoic acid elicit a landing response from *Anopheles gambiae*. *Med Vet Entomol* 14: 195-200.

Hekmat-Scafe DS, Scafe CR, McKinney AJ, and Tanouye MA (2002) Genome-wide analysis of the odorant-binding protein gene family in *Drosophila melanogaster*. *Genome Res* 12: 1357-69.

Himeidan YE, Elbashir MI, and Adam I (2004) Attractiveness of pregnant women to the malaria vector, *Anopheles arabiensis*, in Sudan. *Ann Trop Med Parasitol* 98: 631-3.

Ignell R, Dekker T, Ghaninia M, and Hansson BS (2005) Neuronal architecture of the mosquito deutocerebrum. *J Comp Neurol* 493: 207-40.

Jawara M, Smallegange RC, Jeffries D, Nwakanma DC, Awolola TS, Knols BG, Takken W, and Conway DJ (2009) Optimizing odor-baited trap methods for collecting mosquitoes during the malaria season in The Gambia. *PLoS One* 4: e8167.

Jones WD, Nguyen TA, Kloss B, Lee KJ, and Vosshall LB (2005) Functional conservation of an insect odorant receptor gene across 250 million years of evolution. *Curr Biol* 15: R119-21.

Jones WD, Cayirlioglu P, Kadow IG, and Vosshall LB (2007) Two chemosensory receptors together mediate carbon dioxide detection in Drosophila. *Nature* 445: 86-90.

Kaburi JC, Githuto JN, Muthami L, Ngure PK, Mueke JM, and Mwandawiro CS (2009) Effects of long-lasting insecticidal nets and zooprophylaxis on mosquito feeding behaviour and density in Mwea, central Kenya. *J Vector Borne Dis* 46: 184-90.

Kasili S, Odemba N, Ngere FG, Kamanza JB, Muema AM, Kutima HL (2009) Entomological assessment of the potential for malaria transmission in Kibera slum of Nairobi, Kenya. *J Vector Borne Dis* 46: 273-9.

Kent LB and Robertson HM (2009) Evolution of the sugar receptors in insects. *BMC Evol Biol* 9: 41.

Kent LB, Walden KK, and Robertson HM (2008) The Gr family of candidate gustatory and olfactory receptors in the yellow-fever mosquito *Aedes aegypti. Chem Senses* 33: 79-93.

Kim MS, Repp A, and Smith DP (1998) LUSH odorant-binding protein mediates chemosensory responses to alcohols in *Drosophila melanogaster*. *Genetics* 150: 711-21.

Krieger J, Raming K, Dewer YM, Bette S, Conzelmann S, and Breer H (2002) A divergent gene family encoding candidate olfactory receptors of the moth *Heliothis virescens*. *Eur J Neurosci* 16: 619-28.

Kwon JY, Dahanukar A, Weiss LA, and Carlson JR (2007) The molecular basis of CO2 reception in Drosophila. *Proc Natl Acad Sci U S A* 104: 3574-8.

Kwon HW, Lu T, Rutzler M, and Zwiebel LJ (2006) Olfactory responses in a gustatory organ of the malaria vector mosquito *Anopheles gambiae*. *Proc Natl Acad Sci U S A* 103: 13526-31.

Lacey ES and Cardé RT (2010) Activation, orientation and landing of female *Culex quinquefasciatus* in response to carbon dioxide and odour from human feet: 3-D flight analysis in a wind tunnel. *Med Vet Entomol* 25: 94-103.

Lardeux F, Loayza P, Bouchité B, and Chavez T (2007) Host choice and human blood index of *Anopheles pseudopunctipennis* in a village of the Andean valleys of Bolivia. *Malar J* 22:8.

Larsson MC, Domingos AI, Jones WD, Chiappe ME, Amrein H, et al. (2004) *Or83b* encodes a broadly expressed odorant receptor essential for Drosophila olfaction. *Neuron* 43: 703-14.

Lefèvre T, Gouagna LC, Dabiré KR, Elguero E, Fontenille D, Renaud F, Costantini C, and Thomas F (2010) Beer consumption increases human attractiveness to malaria mosquitoes. *PLoS One* 5: e9546.

Laughlin JD, Ha TS, Jones DN, and Smith DP (2008) Activation of pheromone-sensitive neurons is mediated by conformational activation of pheromone-binding protein. *Cell* 133: 1255-65.

Lindsay S, Ansell J, Selman C, Cox V, Hamilton K, and Walraven G (2000) Effect of pregnancy on exposure to malaria mosquitoes. *Lancet* 355: 1972.

Lundin C, Käll L, Kreher SA, Kapp K, Sonnhammer EL, Carlson JR, Heijne G, and Nilsson I (2007) Membrane topology of the Drosophila OR83b odorant receptor. *FEBS Lett* 581: 5601-4.

Malaney P, Spielman A, and Sachs J (2004) The malaria gap. *Am J Trop Med Hyg* 71: S141–6.

Masse NY, Turner GC, and Jefferis GS (2009) Olfactory information processing in Drosophila. *Curr Biol* 19: R700-13.

Mboera LE, Takken W, and Sambu EZ (2000) The response of *Culex quinquefasciatus* (Diptera: culicidae) to traps baited with carbon dioxide, 1-octen-3-ol, acetone, butyric acid and human foot odour in Tanzania. *Bull Entomol Res* 90: 155–159.

Mboera LEG and Takken W (1999) Odour-mediated host preference of *Culex quinquefasciatus* in Tanzania. *Entomologia Exper et Appl* 92: 83–88.

Mboera LE, Kihonda J, Braks MA, and Knols BG (1998) Short report: Influence of centers for disease control light trap position, relative to a human-baited bed net, on catches of *Anopheles gambiae* and *Culex quinquefasciatus* in Tanzania. *Am J Trop Med Hyg* 59: 595-6.

Mboera LEG, Knols BGJ, Takken W, and della Torre A (1997) The response of *Anopheles gambiae* s.l. and *A. funestus* (Diptera: Culicidae) to tents baited with human odour or carbon dioxide in Tanzania. *Bull Entomol Res* 87: 173-8.

Meijerink J, Braks MA, and van Loon JJ (2001) Olfactory receptors on the antennae of the malaria mosquito *Anopheles gambiae* are sensitive to ammonia and other sweat-borne components. *J Insect Physiol* 47: 455-64.

Meijerink J and van Loon JJ (1999) Sensitivities of antennal olfactory neurons of the malaria mosquito, *Anopheles gambiae*, to carboxylic acids. *J Insect Physiol* 45: 365-73.

Meyerhof W and Korsching S, eds. (2009) **Chemosensory Systems in Mammals, Fishes, and Insects.** Springer.

Michaelakis A, Mihou AP, Couladouros EA, Zounos AK, and Koliopoulos G (2005) Oviposition Responses of *Culex pipiens* to a Synthetic Racemic *Culex quinquefasciatus* Oviposition Aggregation Pheromone. *J Agric Food Chem* 53: 5225–5229.

Molyneux DH and Nantulya VM (2004) Linking disease control programmes in rural Africa: A pro-poor strategy to reach Abuja targets and millenium development goals. *BMJ* 328: 1129–32.

Moon SJ, Lee Y, Jiao Y, and Montell C (2009) A Drosophila gustatory receptor essential for aversive taste and inhibiting male-to-male courtship. *Curr Biol*. 19: 1623-7.

Muriu SM, Muturi EJ, Shililu JI, Mbogo CM, Mwangangi JM, Jacob BG, Irungu LW, Mukabana RW, Githure JI, and Novak RJ (2008) Host choice and multiple blood feeding behaviour of malaria vectors and other Anophelines in Mwea rice scheme, Kenya. *Malar J* 29: 43.

Nakagawa T and Vosshall LB (2009) Controversy and consensus: noncanonical signaling mechanisms in the insect olfactory system. *Curr Opin Neurobiol* 19: 284-92.

Neuhaus EM, Gisselmann G, Zhang W, Dooley R, Stortkuhl K, and Hatt H (2005) Odorant receptor heterodimerization in the olfactory system of *Drosophila melanogaster*. *Nat Neurosci* 8**:** 15-7.

Nikonov AA, Peng G, Tsurupa G, and Leal WS (2002) Unisex pheromone detectors and pheromone-binding proteins in scarab beetles. *Chem Senses* 27: 495-504.

Njiru BN, Mukabana WR, Takken W, and Knols BG (2006) Trapping of the malaria vector *Anopheles gambiae* with odour-baited MM-X traps in semi-field conditions in western Kenya. *Malaria J* 5: 39.

Nuwaha F (2001) The challenge of chloroquine-resistant malaria in sub-Saharan Africa. *Health Policy Plan* 16: 1–12.

Okumu FO, Killeen GF, Ogoma S, Biswaro L, Smallegange RC, Mbeyela E, et al. (2010) Development and humans. *PLoS One* 5: e8951.

Okwa OO, Akinmolayan FI, Carter V, and Hurd H (2009) Transmission dynamics of malaria in four selected ecological zones of Nigeria in the rainy season. *Ann Afr Med* 8:1-9.

Park SK, Shanbhag SR, Wang Q, Hasan G, Steinbrecht RA, and Pikielny CW (2000) Expression patterns of two putative odorant-binding proteins in the olfactory organs of *Drosophila melanogaster* have different implications for their functions. *Cell Tissue Res* 300: 181-92.

Pates HV, Takken W, Stuke K, and Curtis CF (2001a) Differential behaviour of *Anopheles gambiae* sensu stricto (Diptera: Culicidae) to human and cow odours in the laboratory. *Bull Entomol Res* 91: 289–296.

Pates HV, Takken W, Curtis CF, Huisman PW, Akinpelu O, and Gill GS (2001b) Unexpected anthropophagic behaviour in *Anopheles quadriannulatus*. *Med Vet Entomol* 15: 293-8.

Pelletier J, Hughes DT, Luetje CW, and Leal WS (2010) An odorant receptor from the southern house mosquito *Culex pipiens quinquefasciatus* sensitive to oviposition attractants. *PLoS One* 5: e10090.

Picone D, Crescenzi O, Angeli S, Marchese S, Brandazza A, Ferrara L, Pelosi P, and Scaloni A (2001) Bacterial expression and conformational analysis of a chemosensory protein from *Schistocerca gregaria*. *Eur J Biochem* 268: 4794-801.

Pitts RJ, Fox AN, and Zwiebel LJ (2004) A highly conserved candidate chemoreceptor expressed in both olfactory and gustatory tissues in the malaria vector, *Anopheles gambiae. Proc Natl Acad Sci U S A* 101**:** 5058-63.

Pitts RJ and Zwiebel LJ (2006) Antennal sensilla of two female Anopheline sibling species with differing host ranges. *Malar J* 5:26.

Qiao H, He X, Schymura D, Ban L, Field L, Dani FR, Michelucci E, Caputo B, Torre AD, Iatrou K, Zhou JJ, Krieger J, and Pelosi P (2010) Cooperative interactions between odorant-binding proteins of *Anopheles gambiae*. *Cell Mol Life Sci* Oct 19. [Epub ahead of print]

Qiu YT, Smallegange RC, VAN Loon JJ, and Takken W (2010) Behavioural responses of *Anopheles gambiae* sensu stricto to components of human breath, sweat and urine depend on mixture composition and concentration. *Med Vet Entomol* Nov 25. [Epub ahead of print]

Qiu YT, Smallegange RC, Ter BC, Spitzen J, Van Loon JJ, Jawara M, Milligan P, Galimard AM, Van Beek TA, Knols BG, and Takken W (2007) Attractiveness of MM-X traps baited with human or synthetic odor to mosquitoes (Diptera: Culicidae) in The Gambia. *J Med Entomol* 44: 970-83.

Qiu YT, Smallegange RC, Van Loon JJ, Ter Braak CJ, and Takken W (2006) Interindividual variation in the attractiveness of human odours to the malaria mosquito *Anopheles gambiae* s.s. *Med Vet Entomol* 20: 280-7.

Qiu YT, Smallegange RC, Hoppe S, van Loon JJ, Bakker EJ, and Takken W (2004) Behavioural and electrophysiological responses of the malaria mosquito *Anopheles gambiae* Giles sensu stricto (Diptera: Culicidae) to human skin emanations. *Med Vet Entomol* 18: 429-38.

Ray A, van Naters WG, Shiraiwa T, and Carlson JR (2007) Mechanisms of odor receptor gene choice in Drosophila. *Neuron* 53: 353-69.

Ray A, van der Goes van Naters W, and Carlson JR (2008) A regulatory code for neuron-specific odor receptor expression. *PLoS Biol* 6: e125.

Riehle MM, Guelbeogo WM, Gneme A, Eiglmeier K, Holm I, Bischoff E, Garnier T, Snyder GM, Li X, Markianos K, Sagnon N, and Vernick KD (2011) A cryptic subgroup of *Anopheles gambiae* is highly susceptible to human malaria parasites. *Science* 331: 596-8.

Riffell JA, Lei H, Christensen TA, and Hildebrand JG (2009) Characterization and coding of behaviorally significant odor mixtures. *Curr Biol* 19: 335-40.

Riviere S, Lartigue A, Quennedey B, Campanacci V, Farine JP, Tegoni M, Cambillau C and Brossut R (2003) A pheromone-binding protein from the cockroach *Leucophaea maderae*: Cloning, expression and pheromone binding. *Biochem J* 371**:** 573-9.

Robertson HM, Gadau J, and Wanner KW (2010) The insect chemoreceptor superfamily of the parasitoid jewel wasp *Nasonia vitripennis*. *Insect Mol Biol* 19 Suppl 1: 121-36.

Robertson HM and Kent LB (2009) Evolution of the gene lineage encoding the carbon dioxide receptor in insects. *J Insect Sci* 9:19.

Robertson HM and Wanner KW (2006) The chemoreceptor superfamily in the honey bee, *Apis mellifera*: expansion of the odorant, but not gustatory, receptor family. *Genome Res* 16: 1395-403

Robertson HM, Warr CG, and Carlson JR (2003) Molecular evolution of the insect chemoreceptor gene superfamily in *Drosophila melanogaster*. *Proc Natl Acad Sci U S A* 100 Suppl 2: 14537-42.

Rudolfs W (1922) Chemotropism of Mosquitoes. *Bull New Jersey Agric Exp Sta* 367: 1-26

Russell S (2004) The economic burden of illness for households in developing countries: A review of studies focusing on malaria, tuberculosis,

and human immunodeficiency virus/acquired immunodeficiency syndrome. *Am J Trop Med Hyg* 71: S147-55.

Rützler M and Zwiebel LJ (2005) Molecular biology of insect olfaction: recent progress and conceptual models. *J Comp Physiol A Neuroethol Sens Neural Behav Physiol* 191: 777-90.

Sabbatani S, Fiorino S, and Manfredi R (2010) The emerging of the fifth malaria parasite (*Plasmodium knowlesi*): a public health concern? *Braz J Infect Disease* 14: 299-309.

Sachs J and Malaney O (2002) The economic and social burden of malaria. *Nature* 415: 680-5.

Sakurai T, Nakagawa T, Mitsuno H, Mori H, Endo Y, Tanoue S, Yasukochi Y, Touhara K and Nishioka T (2004) Identification and functional characterization of a sex pheromone receptor in the silkmoth *Bombyx mori*. *Proc Natl Acad Sci U S A* 101**:** 16653-8.

Sánchez-Gracia A, Vieira FG, and Rozas J (2009) Molecular evolution of the major chemosensory gene families in insects. *Heredity* 103: 208-16.

Sato K and Touhara K (2009) Insect olfaction: receptors, signal transduction, and behavior. *Results Probl Cell Differ* 47: 121-38.

Sato K, Pellegrino M, Nakagawa T, Nakagawa T, Vosshall LB, and Touhara K (2008) Insect olfactory receptors are heteromeric ligand-gated ion channels. *Nature*. 452:1002-6.

Schmied WH, Takken W, Killeen GF, Knols BG, and Smallegange RC (2008) Evaluation of two counterflow traps for testing behaviour-mediating compounds for the malaria vector *Anopheles gambiae* s.s. under semi-field conditions in Tanzania. *Malar J* 7: 230.

Schymura D, Forstner M, Schultze A, Kröber T, Swevers L, Iatrou K, Krieger J (2010). Antennal expression pattern of two olfactory receptors and an odorant binding protein implicated in host odor detection by the malaria vector *Anopheles gambiae*. *Int J Biol Sci* 2010 6: 614-26.

Shanbhag SR, Hekmat-Scafe D, Kim MS, Park SK, Carlson JR, Pikielny C, Smith DP, and Steinbrecht RA (2001) Expression mosaic of odorant-binding proteins in Drosophila olfactory organs. *Microsc Res Tech* 55: 297-306.

Sherman IW (2007) **Twelve Diseases that Changed Our World.** ASM Press.

Sinka ME, Bangs MJ, Manguin S, Coetzee M, Mbogo CM, Hemingway J, et al. (2010) The dominant Anopheles vectors of human malaria in Africa, Europe and the Middle East: occurrence data, distribution maps and bionomic précis. *Parasit Vectors* 3: 117.

Slone J, Daniels J, and Amrein H (2007) Sugar receptors in Drosophila. *Curr Biol* 17: 1809-16.

Smadja C, Shi P, Butlin RK, and Robertson HM (2009) Large gene family expansions and adaptive evolution for odorant and gustatory receptors in the pea aphid, *Acyrthosiphon pisum*. *Mol Biol Evol* 26: 2073-86.

Smallegange RC, Knols BG, and Takken W (2010) Effectiveness of synthetic versus natural human volatiles as attractants for *Anopheles gambiae* (Diptera: Culicidae) sensu stricto. *J Med Entomol* 47: 338-44.

Smallegange RC, Verhulst NO, and Takken W (2011). Sweaty skin: an invitation to bite? *Trends Parasitol* Jan 19 [Epub ahead of print].

Smallegange RC, Qiu YT, Bukovinszkin-Kiss G, Van Loon JJ, and Takken W (2009) The effect of aliphatic carboxylic acids on olfaction-based host-seeking of the malaria mosquito *Anopheles gambiae* sensu stricto. *J Chem Ecol* 35: 933-43.

Smallegange RC, Qiu YT, van Loon JJ, and Takken W (2005) Synergism between ammonia, lactic acid and carboxylic acids as kairomones in the host-seeking behaviour of the malaria mosquito *Anopheles gambiae* sensu stricto (Diptera: Culicidae). *Chem Senses* 30: 145-52.

Smith CR, Smith CD, Robertson HM, Helmkampf M, Zimin A, Yandell M, Holt C, et al. (2011) Draft genome of the red harvester ant *Pogonomyrmex barbatus*.
Proc Natl Acad Sci U S A Jan 31. [Epub ahead of print]

Sousa CA, Pinto J, Almeida AP, Ferreira C, do Rosário VE, and Charlwood JD (2001) Dogs as a favored host choice of *Anopheles gambiae* sensu stricto (Diptera: Culicidae) of São Tomé West Africa. *J Med Entomol* 38:122-5.

Takken W and Knols BGJ, eds. (2009) **Olfacation in Vector-Host Interactions: Ecology and Control of Vector Borne Diseases**. Wageningen Academic.

Tanga MC, Ngundu WI, and Tchouassi PD (2011) Daily survival and human blood index of major malaria vectors associated with oil palm

cultivation in Cameroon and their role in malaria transmission. *Trop Med Int Health* Jan 19. [Epub ahead of print]

Thorne N, Bray S, and Amrein H (2005) Function and expression of the Drosophila *gr* genes in the perception of sweet, bitter and pheromone compounds. *Chem Senses*. 30 Suppl 1: i270-2.

Tichy AL, Ray A, and Carlson JR (2008) A new Drosophila POU gene, *pdm3*, acts in odor receptor expression and axon targeting of olfactory neurons. *J Neurosci* 28: 7121-9.

Touhara K and Vosshall LB (2009) Sensing odorants and pheromones with chemosensory receptors. *Annu Rev Physiol* 71: 307-32.

Verhulst NO, Mbadi PA, Kiss GB, Mukabana WR, van Loon JJ, Takken W, and Smallegange RC (2011) Improvement of a synthetic lure for *Anopheles gambiae* using compounds produced by human skin microbiota. *Malar J* 10: 28.

Verhulst NO, Andriessen R, Groenhagen U, Bukovinszkin Kiss G, Schulz S, Takken W, van Loon JJ, Schraa G, and Smallegange RC (2010) Differential attraction of malaria mosquitoes to volatile blends produced by human skin bacteria. *PLoS One*. 5: e15829.

Verhulst NO, Beijleveld H, Knols BG, Takken W, Schraa G, Bouwmeester HJ, and Smallegange RC (2009) Cultured skin microbiota attracts malaria mosquitoes. *Malar J* 8: 302.

Verhulst NO, Takken W, Dicke M, Schraa G, and Smallegange RC (2010) Chemical ecology of interactions between human skin microbiota and mosquitoes. *FEMS Microbiol Ecol* 74: 1-9.

Vosshall LB, Amrein H, Morozov PS, Rzhetsky A, and Axel R (1999) A spatial map of olfactory receptor expression in the Drosophila antenna. *Cell* 96: 725-36.

Vogt RG and Riddiford LM (1981) Pheromone binding and inactivation by moth antennae. *Nature* 293: 161-3.

Vogt RG, Callahan FE, Rogers ME, and Dickens JC (1999) Odorant binding protein diversity and distribution among the insect orders, as indicated by lap, an obp-related protein of the true bug *Lygus lineolaris* (Hemiptera, Heteroptera). *Chem Senses* 24**:** 481-95.

Vogt RG, Kohne AC, Dubnau JT, and Prestwich GD (1989) Expression of pheromone binding proteins during antennal development in the gypsy moth *Lymantria dispar*. *J Neurosci* 9**:** 3332-46.

Vogt RG, Prestwich GD, and Lerner MR (1991) Odorant-binding-protein subfamilies associate with distinct classes of olfactory receptor neurons in insects. *J Neurobiol* 22**:** 74-84.

Wang G, Qiu YT, Lu T, Kwon HW, Pitts RJ, van Loon JJ, Takken W, and Zwiebel LJ (2009) *Anopheles gambiae* TRPA1 is a heat-activated channel expressed in thermosensitive sensilla of female antennae. *Eur J Neurosci* 30: 967-74.

Wang G, Carey AF, Carlson JR, and Zwiebel LJ (2010) Molecular basis of odor coding in the malaria vector mosquito *Anopheles gambiae*. *Proc Natl Acad Sci U S A* 107: 4418-23.

Wanner KW, Anderson AR, Trowell SC, Theilmann DA, Robertson HM, and Newcomb RD (2007) Female-biased expression of odourant receptor genes in the adult antennae of the silkworm, *Bombyx mori*. *Insect Mol Biol* 16: 107-19.

Weiss LA, Dahanukar A, Kwon JY, Banerjee D, and Carlson JR (2011) The molecular and cellular basis of bitter taste in Drosophila. *Neuron* 69: 258-72.

Wetzel CH, Behrendt H, Gisselmann G, Storkuhl KF, Hovemann B, and Hatt H (2001) Functional expression and characterization of a *drosophila* odorant receptor in a heterologous cell system. *PNAS* 98**:** 9377-9380.

Wicher D, Schafer R, Bauernfeind R, Stensmyr MC, Heller R, et al. (2008) Drosophila odorant receptors are both ligand-gated and cyclic-nucleotide-activated cation channels. *Nature* 452: 1007–11.

World Health Organization (2002) **Scaling-up Insecticide-Treated Netting Programmes in Africa.**

World Health Organization (2003) **Africa Malaria Report 2003**.

World Health Organization (2010) **World Malaria Report 2010**.

Xia Y and Zwiebel LJ (2006) Identification and characterization of an odorant receptor from the West Nile virus mosquito, *Culex quinquefasciatus*. *Insect Biochem Mol Biol* 36: 169-76.

Xia Y, Wang G, Buscariollo D, Pitts JR, Wenger H, and Zwiebel LJ (2008) The molecular basis of olfactory-based behavior in *Anopheles gambiae* larvae. *Proc Natl Acad Sci U S A* 105: 6433–38.

Xu PX, Zwiebel LJ, and Smith DP (2003) Identification of a distinct family of genes encoding atypical odorant-binding proteins in the malaria vector mosquito, *Anopheles gambiae Insect Mol Biol* 12: 549-60.

Xu P, Atkinson R, Jones DN, and Smith DP (2005) Drosophila OBP LUSH is required for activity of pheromone-sensitive neurons. *Neuron* 45: 193-200.

Yamey G (2004) Roll Back Malaria: A failing global health campaign. *BMJ* 328: 1086–7.

CHAPTER II

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

Preface

The following publication by Liu et al. appeared in the journal *PLoS Biology* in 2010 (Volume 8, No. 8, pii: e1000467). I was a co-first author of this publication based on my contributions to the work contained therein. My experimental contributions included identifying and annotating the large family of 46 *An. gambiae* Ionotropic Receptors (*AgIrs*) plus 9 ionotropic glutamate receptors, examining their expression patterns in chemosensory tissues of adults and larvae, synthesizing *AgIr76b* siRNA. In addition, I contributed to all aspects of the experimental design, statistical analyses, figures, and manuscript writing. My own unpublished data (Table 2 and Figure 12) have been added to this chapter that describe in more detail the expression of *AgIrs* in adult chemosensory tissues.

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 malaria (Takken and Knols 1999). Significant progress has been made in identifying the components of olfactory pathways in *An. gambiae* (Fox et al. 2001; Merrill et al. 2003; Hallem et al. 2004; Pitts et al. 2004; Kwon et al. 2006). 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; Hill et al. 2002), several studies have disputed the GPCR nature of Anopheline and other insect ORs (Benton et al. 2006; Lundin et al. 2007; Sato et al. 2008; Wicher et al. 2008; Smart et al. 2008), which likely form ligand-gated heteromeric ion channels that activate ORNs through ionotropic (Sato et al. 2008; Smart et al. 2008) as well as perhaps metabotropic mechanisms (Wicher et al. 2008). In addition, members of a family of another set of chemosensory receptors related to ionotropic glutamate receptors have recently been described in *Drosophila melanogaster* (Benton et al. 2009). 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 OR83b-like protein together with a conventional OR that presumably mediates odorant binding specificity (Hallem et al. 2004;

Benton et al. 2006; Larsson et al. 2004). In *An. gambiae*, 73 of the 79 AgORs originally identified (Hill et al. 2002) are expressed in the adult and 13 are expressed in larval stages (Xia et al. 2008). The non-conventional Anopheline OR83b-like family member, AgOR7, is widely expressed in nearly all olfactory sensilla with the notable exception of grooved-peg sensilla (Pitts et al. 2004), which are activated in vivo by compounds such as ammonia, lactic acid, and other carboxylic acids that are major components of human sweat (Cork and Park 1996; Bernier et al. 2000) known to evoke physiological and/or behavioral activity in *An. gambiae* (Carey et al. 2010; Wang et al. 2010). 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 (Carey et al. 2010; Wang et al. 2010).

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 ORNs (Xia et al. 2008). 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 (Xia et al. 2008). 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.

Methods

Mosquito Rearing

An. gambiae sensu stricto, originated from Suakoko, Liberia, was reared as described (Fox et al. 2001). 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* 2nd or 3rd 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 the highest grade commercially available) in pre-heated (70 degrees C) 2% NuSieve, GTG low-melting-temperature agarose (Cambrex Bio Science). The assay was performed in a 10x1.5 cm Petri dish containing 50 ml of 27 degrees 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 1s 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 degrees 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 degrees, 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 degrees 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: 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-2 and stage-3 larvae, we calculated the average larval body length as approximately 3.25 mm in our CCD system, thereby establishing a threshold for larval movements at approximately 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

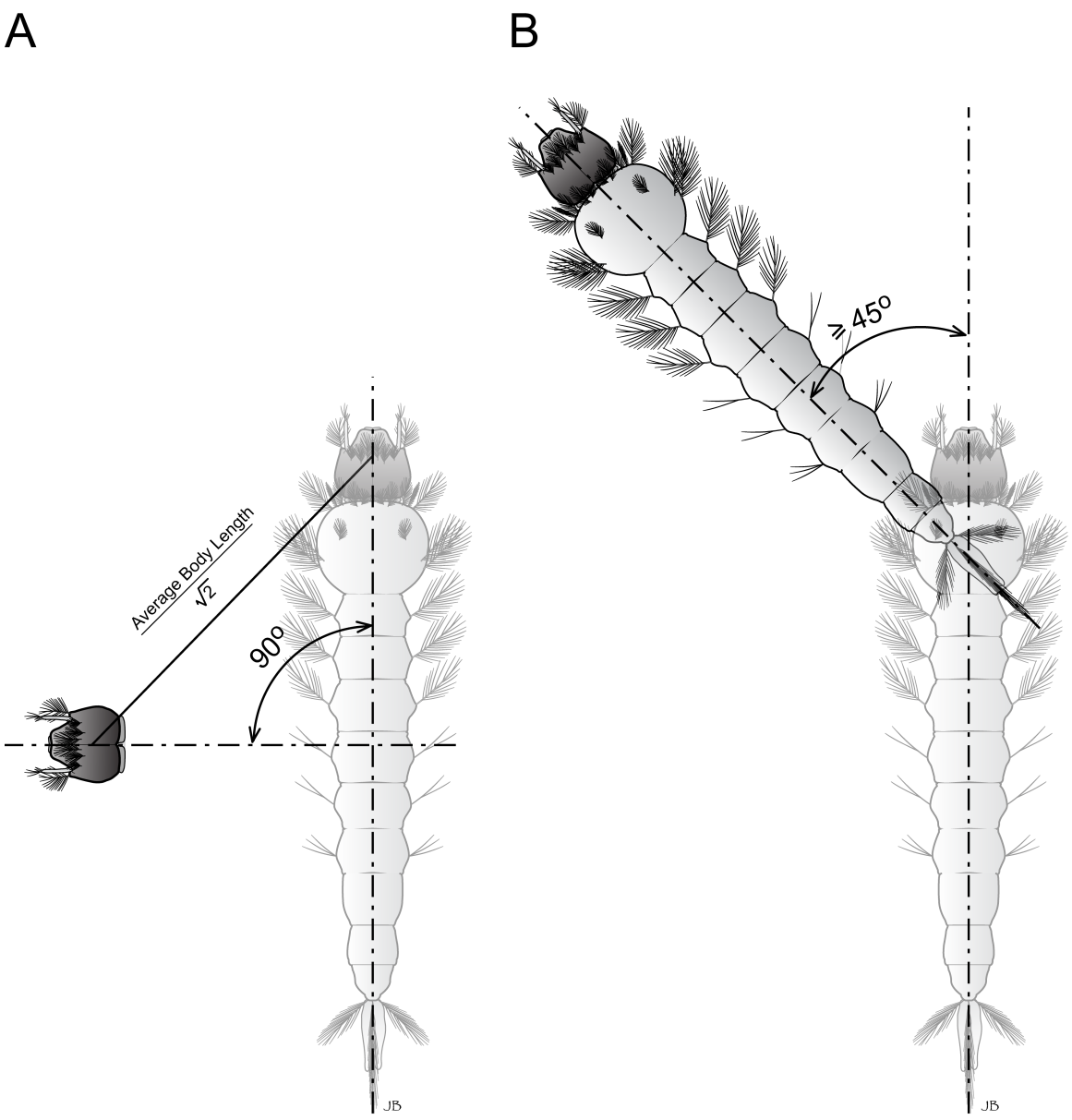


Figure 1. Operational definitions of larval movements and turns. (A) A larval body movement threshold is characterized by a larva turning its body axis by 90 degrees and its head traveling the distance indicated. (B) A larval turn threshold is defined by a 45 degree angle between two successive larval tracking vectors.

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 2nd instar stages, after which larvae were allowed to recover for 24h prior to behavioral testing.

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 method (Saitou and Nei 1987) 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

(Technical University of Denmark, 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 described (Fox et al. 2001). PCR primers specific for *AgIrs* were as follows: *AgIr8a*: f5'-CCCTATGAGTGCAGAAAATT-3' and r5'-GGTACAGCACGTCTTCTGCG-3'; *AgIr25a*: f5'-CAACCGACATACGCTACCAA-3' and r5'-ACGATGAATACGCCTCCGAT-3'; AgIr41a: f5'-ACTGG- GAACTGGAGGTGGTG-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'-GTAGCTGCTGCAAACTTCGG-3'. cDNA amplicons were TOPO-TA cloned into plasmid pCRII (Invitrogen) and sequenced to confirm their identities.

Expression of AgIrs in adult antennae and maxillary palps were determined by RNA-seq analysis as described in Chapter III (Methods) of

this dissertation. *In-situ* hybridizations were carried out as described (Kwon et al. 2006), except that frozen sections were used instead of paraffin sections.

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 RNAse III digestion using Silencer siRNA Construction reagents and protocols (Applied BioSystems/Ambion, Austin, TX). Healthy, wild-type 2nd instar *An. gambiae* larvae were chosen for micro-injection. They were pre-immobilized on 3MM filter paper on top of a 4 degrees C chill platform (BioQuip Inc, Rancho Dominquez, 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 degrees C distilled water with 1 ml of

larval food (as described in Mosquito Rearing section) for 48h. Larvae were monitored every 24h post-injection, and non-viable individuals were discarded.

Real-Time PCR (qRT-PCR)

Subsequent to experimental treatments and behavioral assays, *AgOr7*, *AgOr40* and *AgIr76b* transcript levels were determined by means of quantitative RT-PCR. Each sample was comprised of 10 (*AgOr7*) 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'-GTAGCTGCTGCAAACTTCGG-3' (product size: 458bp cDNA); *AgOr7*, f5'-ATCTTTGGCAATCGGCTCATC-3' and r5'-GGCTCCAAGAACCGAAGC-3' (product size: 346 bp cDNA); *AgOr40*, f5'-GACCCTCAAGAACCAGGGCT-3' and r5'-AATGATGGTGTAGTACGAGAAGG-3'; *AgIr76b*, f5'-AT-

CTTCGATCCAGAGTTGCT-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 µl and 2 µl 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 Pfaffl (2001).

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 system (Xia et al. 2008). 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).

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 paradigm (Xia et al. 2008). 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 (Xia et al. 2008). 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

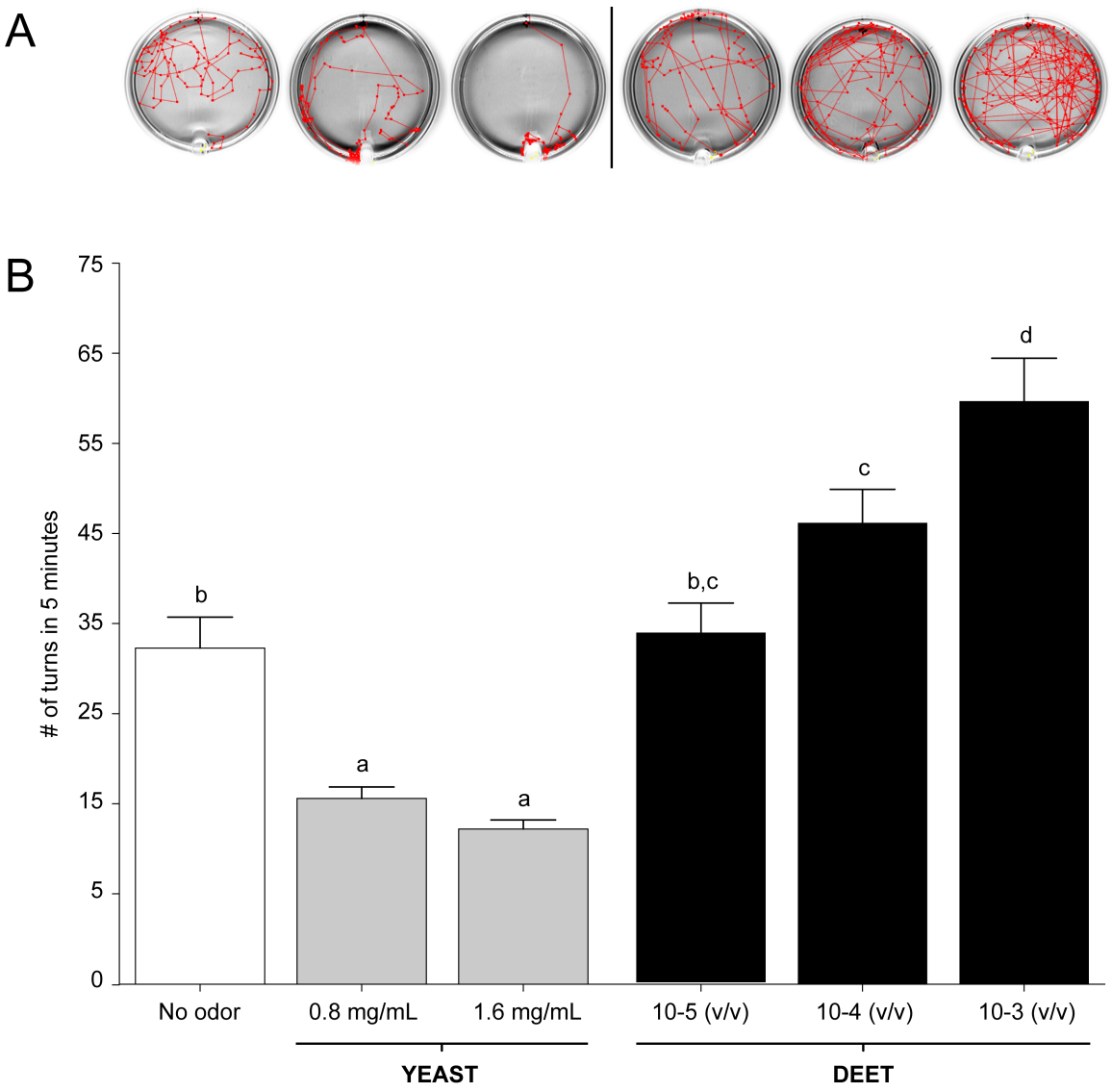


Figure 2. Larval responses in *An. gambiae* to yeast and DEET elicit opposite behaviors. (A) 2-D tracking maps (top view) of freely moving individual larva during a 5 min time lapse. (B) Average number of turns exhibited by larvae in response to no odor, two concentrations of yeast paste, and three concentrations 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 +/- SE, n = 10.

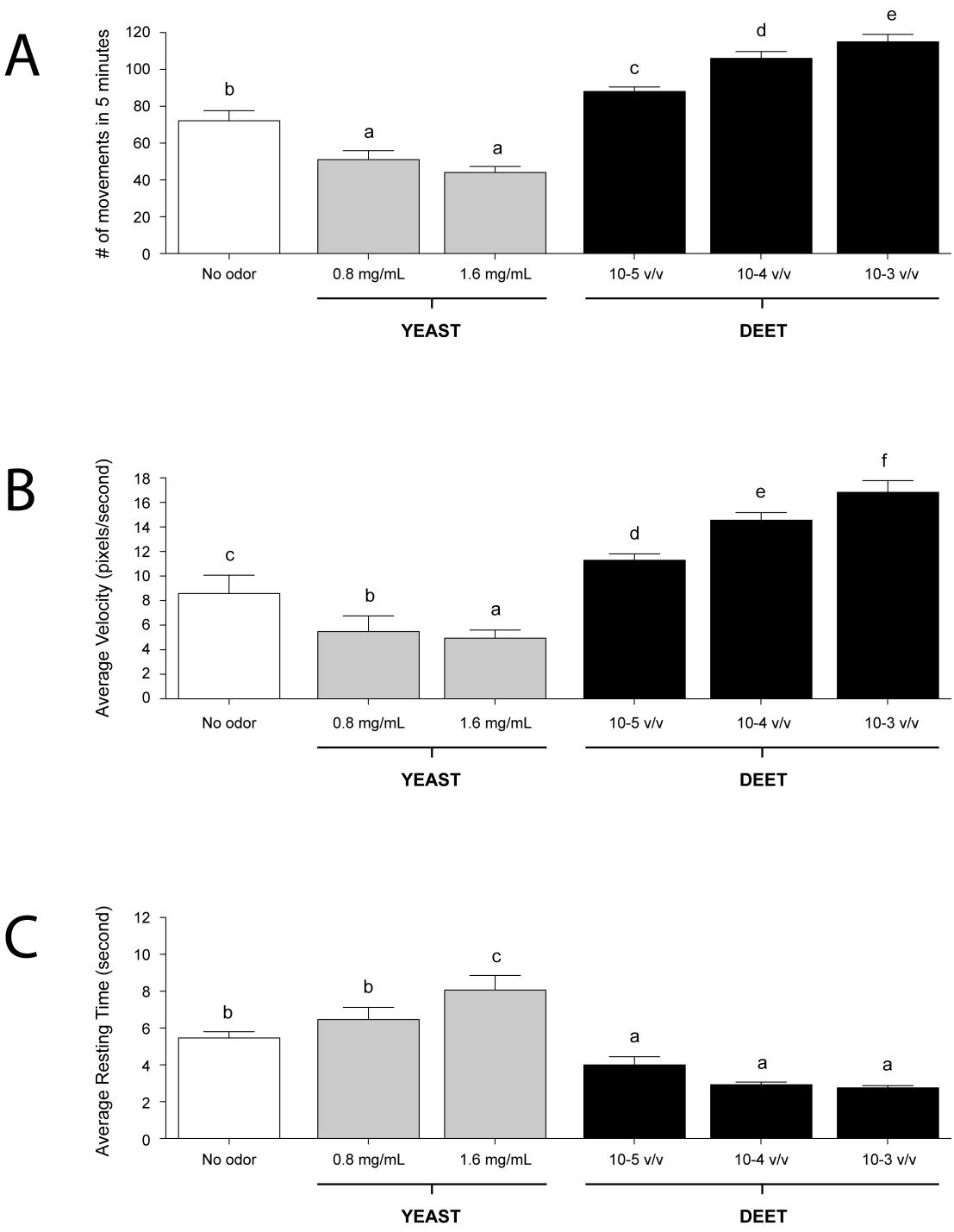


Figure 3. Behavioral effects of yeast and DEET on *An. gambiae*. Larval responses to yeast and DEET stimuli. Average number of movements (A), velocity (B), and resting time (C)—histograms of larval responses to two concentrations of yeast paste and three concentrations of DEET. Compared with the no-odor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

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.

AgOR 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 *AgOr7*, which is the *An. gambiae* ortholog of the non-conventional *Drosophila* OR, *DmOr83b* (Pitts et al. 2004; Hill et al. 2002), and is highly expressed in the larval antenna (Xia et al. 2008). In the absence of effective strategies to generate mutant or transgenic strains of *An. gambiae*, we used RNA

interference (RNAi) to reduce *AgOr7* 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 *AgOr7* 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 *AgOr7* 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 *AgOr7* resulted in dramatic decreases in levels of this transcript.

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

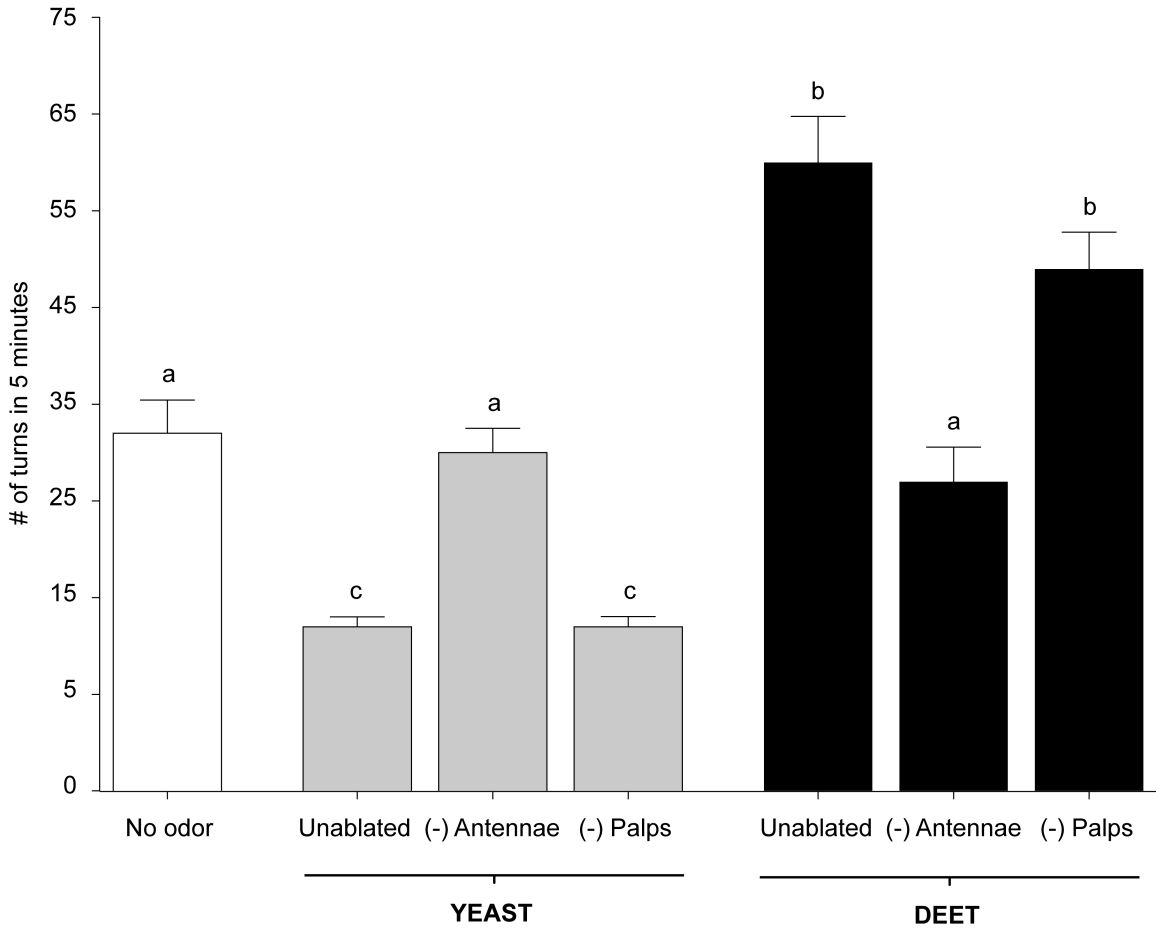
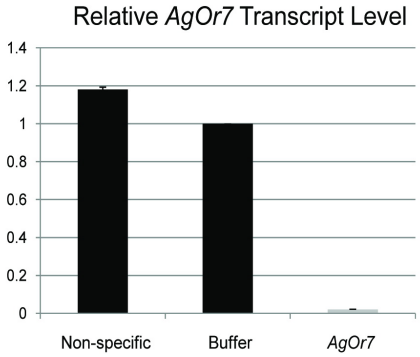


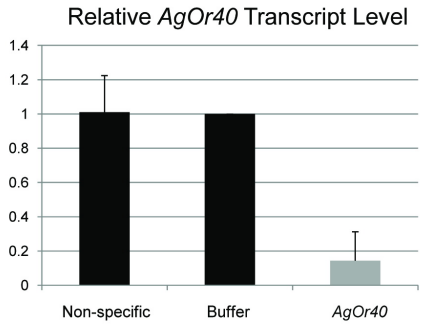
Figure 4. Larval antennae mediate responses to yeast and DEET. In the presence of yeast and DEET, unablated and palp-ablated larvae responded equally to both; ablation of the antennae, however, significantly increased or decreased the number of turns ($p<0.05$) in response to yeast and DEET, respectively.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

microinjection with *AgOr7* or control siRNAs. In contrast, a 1x10-3 (v/v) dilution of DEET in individuals that received *AgOr7* 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 *AgOr7*-dependent whilst larval responses to yeast paste are *AgOr7*-independent. Functional studies using *Xenopus* oocytes (Xia et al. 2008) 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 *AgOr7* 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 *AgOr7* silencing, qRT-PCR studies were carried out on experimental and control larvae after behavioral testing to assess the levels of *AgOr40*



Treatment	*AgOr7* mRNA level fold change		
Non-specific siRNA inject	1.14	1.18	1.22
Buffer inject	1	1	1
AgOr7 siRNA inject	1/40.5	1/52	1/50

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



Treatment	*AgOr40* mRNA level fold change		
Non-specific siRNA inject	0.77	1.17	1.10
Buffer inject	1	1	1
AgOr40 siRNA inject	1/11.7	1/2.4	1/5.1

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

Figure 5. Quantitative RT-PCR analysis demonstrates significant transcript level reduction of *AgOr7* and *AgOr40* after siRNA treatment. Larval cDNAs for qRT-PCR were generated using equal amounts (2 µg for *AgOr7* 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. *AgOr7* and *AgOr40* mRNA levels were quantified as fold-changes relative to *rps7* using the method of Pfaffl (2001). *AgOr7* and *AgOr40* levels are shown after normalization to buffer-alone controls in each of three experimental replicates. Histograms showing averaged AgOr7 and AgOr40 levels normalized to buffer-alone injection controls. Standard errors were +/-0.041 and +/-0.029 for non-specific and *AgOr7* 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.

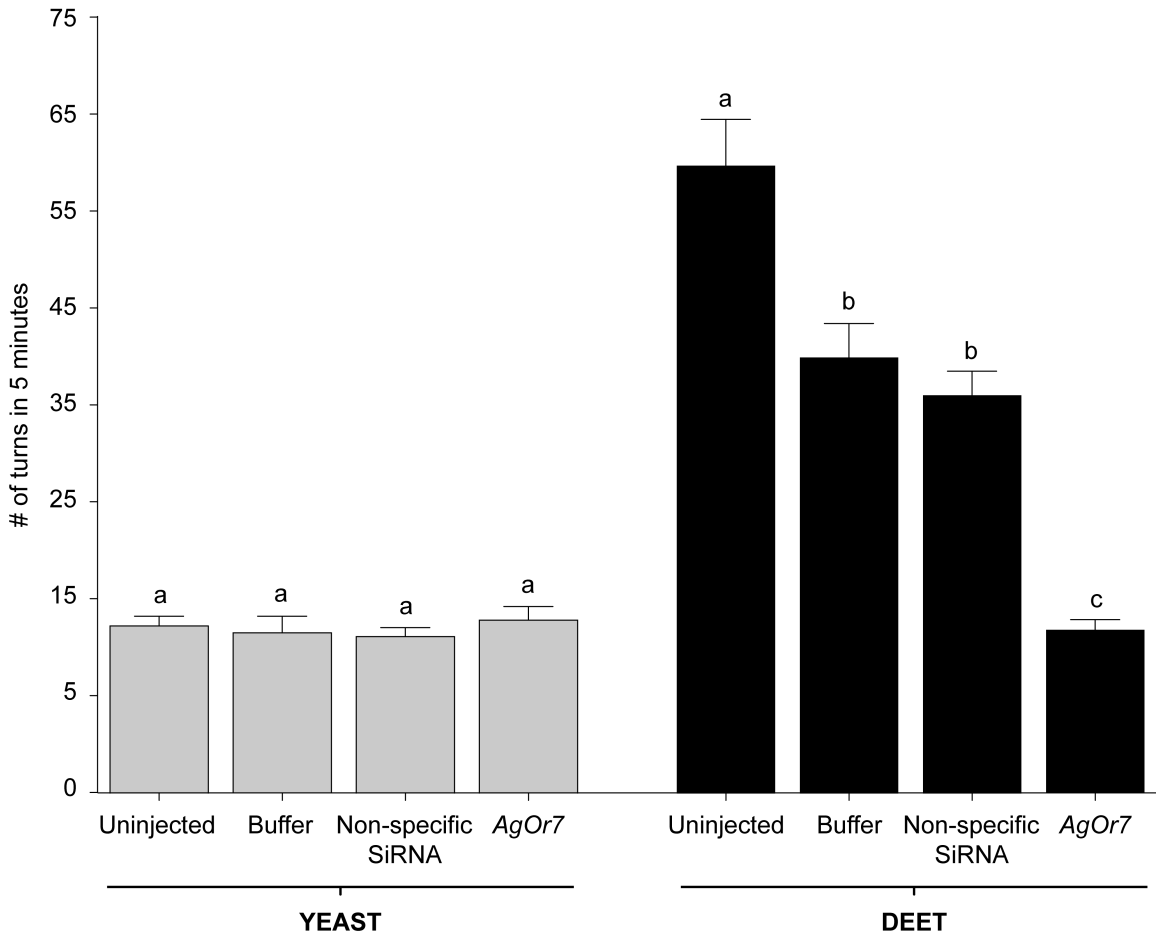


Figure 6. Differential sensitivity of larval responses in *An. gambiae* to siRNA-mediated knockdown of *AgOr7* is odorant dependent. The average number of turns exhibited by uninjected larvae as well as those receiving mock (buffer-alone), non-specific, or siRNA injections in response to yeast paste 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 *AgOr7* 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 +/- SE, n = 10.

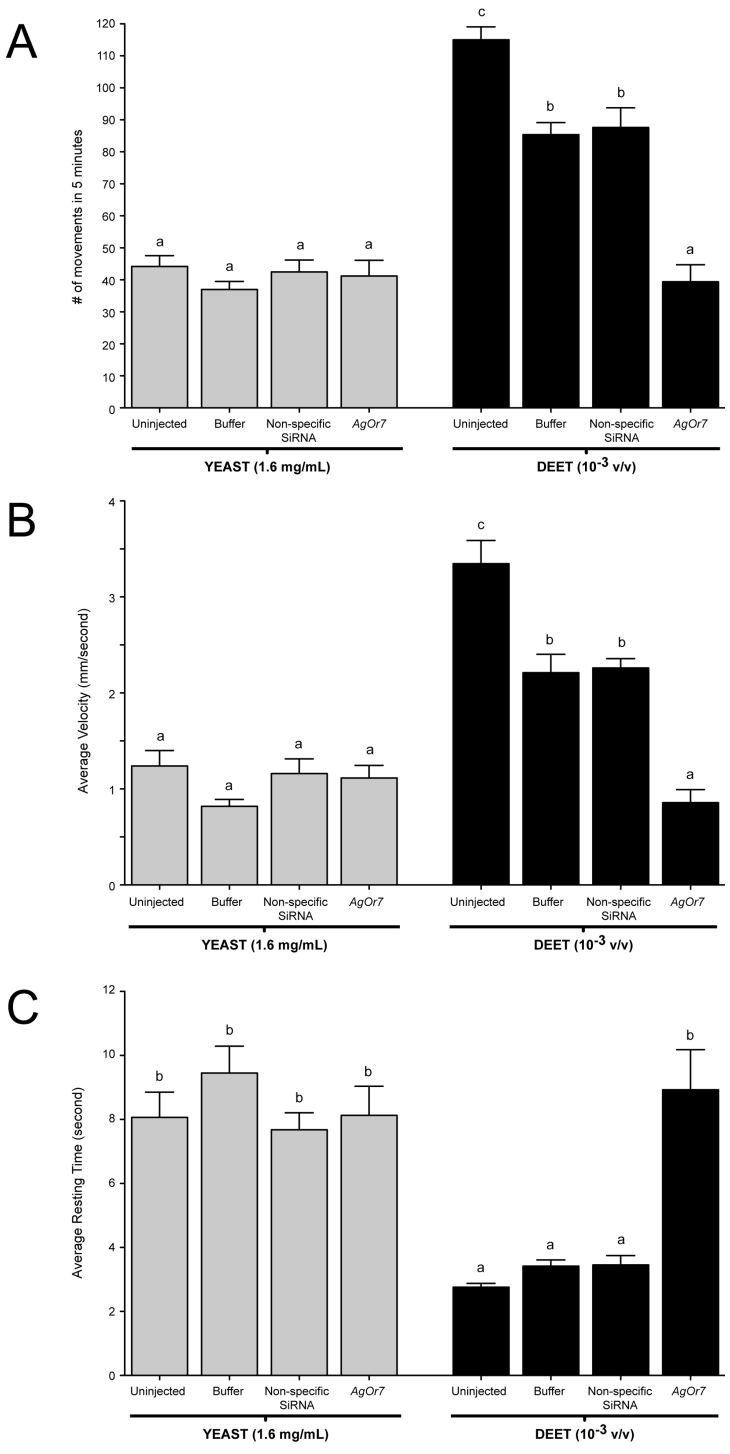

A
# of movements in 5 minutes
120
110
100
90
80
70
60
50
40
30
20
10
0
a
a
a
a
c
b
b
a
Uninjected
Buffer
Non-specific SiRNA
AgOr7
Uninjected
Buffer
Non-specific SiRNA
AgOr7
YEAST (1.6 mg/mL)
DEET (10-3 v/v)
B
Average Velocity (mm/second)
4
3
2
1
0
a
a
a
a
c
b
b
a
Uninjected
Buffer
Non-specific SiRNA
AgOr7
Uninjected
Buffer
Non-specific SiRNA
AgOr7
YEAST (1.6 mg/mL)
DEET (10-3 v/v)
C
Average Resting Time (second)
12
10
8
6
4
2
0
b
b
b
b
a
a
a
b
Uninjected
Buffer
Non-specific SiRNA
AgOr7
Uninjected
Buffer
Non-specific SiRNA
AgOr7
YEAST (1.6 mg/mL)
DEET (10-3 v/v)

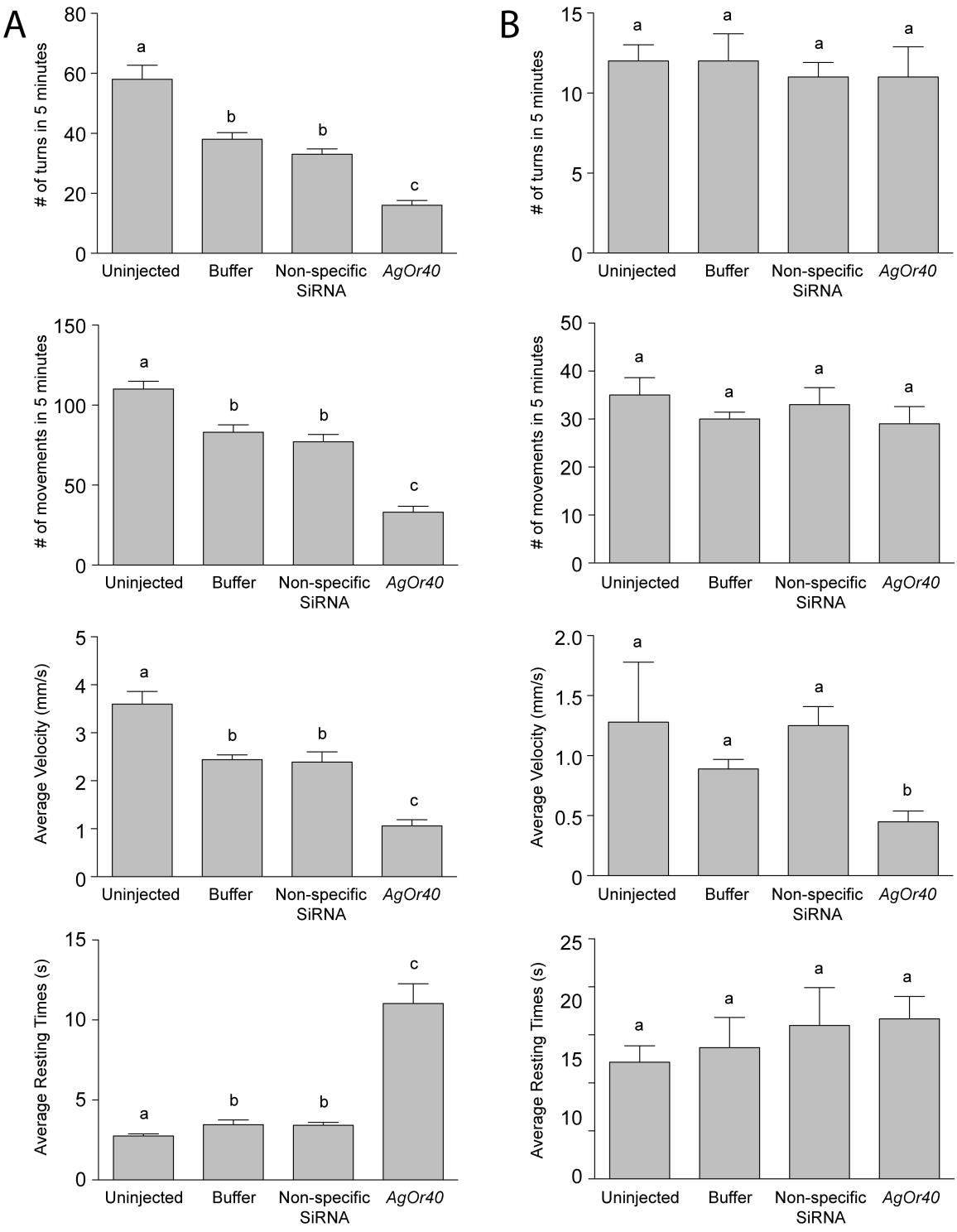
Figure 7. Larval behaviors after injection of non-specific small interfering RNA (siRNA). Averaged responses of buffer, non-specific, and *AgOr7* siRNA-injected larvae in the presence of 1.6 mg/ml yeast paste and a 10-3 v/v dilution of DEET. Larval movement (A), velocity (B), and resting time (C) behaviors of larvae in response to yeast paste and DEET. Knockdown of *AgOr7* 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 +/- SE, n = 10.

transcripts. These data (Figure 5) confirm that microinjection of siRNAs targeting *AgOr40* resulted in dramatic decreases in *AgOr40* transcript levels without significantly altering *AgOr7* 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*, but does not rule out the involvement of other AgORs, especially in adults.

AgIRs Mediate AgOR Independent Olfactory Responses

Based on the *AgOr7*-independent response of larvae to yeast paste, we next investigated whether *AgOr7*-dependent and -independent olfactory signaling exists in *An. gambiae* larvae. In doing so, we considered that *AgOr7* independence of the larval yeast response might, in part, reflect that yeast paste is a complex mixture, some components of which may activate *AgOr7*- 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 assays (Xia et al. 2008).

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.*


A
B
# of turns in 5 minutes
# of movements in 5 minutes
Average Velocity (mm/s)
Average Resting Times (s)
Uninjected
Buffer
Non-specific SiRNA
AgOr40

.

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 +/- SE, n = 10.

melanogaster (DmIRs) (Benton et al. 2009). 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 genes (Croset et al. 2010) 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 and their chromosome positions is given in Table 1.

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 (for review see Mayer 2006).

Interestingly, two of the strongest AgIR homologs of DmIRs are found within the iGluR clade (Figure 9). 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* (Benton et al. 2009). 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 (Mayer 2006). Importantly, some classes of NMDA receptors also lack the 3rd residue (Mayer 2006). 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 (unpublished data).

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 *AgIr* 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 *AgIrs* 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). Moreover, broad expression of *AgIrs* in adult *An. gambiae* chemosensory tissues was observed (Table 2). *In-situ* hybridization using an *AgIr25a*-specific

Gene	peptide name	VectorBase ID	Chromosome: base pairs (strand)
AgamGLURI	AgGLURI	AGAP006027	2L: 25,274,226-25,314,781 (+)
AgamGLURIIa	AgGLURIIa	AGAP000803	X: 14,739,086-14,744,472 (+)
AgamGLURIIb	AgGLURIIb	AGAP000801	X: 14,618,285-14,702,072 (-)
AgamGLURIIc	AgGLURIIc	AGAP000798	X: 14,600,998-14,612,301 (-)
AgamGLURIId	AgGLURIId	AGAP002797	2R: 27,638,116-27,644,376 (-)
AgamGLURIIe	AgGLURIIe	AGAP012447	unk: 1,553,896-1,557,262 (+)
AgamNMDAR1	AgNMDAR1	AGAP001478	2R: 5,390,305-5,394,755 (+)
AgamNMDAR2	AgNMDAR2	AGAP012429	unk: 284,129-400,942 (-)
AgamNMDAR3	AgNMDAR3	AGAP005527	2L: 16,640,867-16,647,151 (-)
AgamGLUvir7h.1	AgIR7h.1	AGAP013154	X: 12,751,181-12,753,181 (-)
AgamGLUvir7i	AgIR7i	AGAP013363	2R: 26,793,376-26,795,314 (+)
AgamGLUvir7n	AgIR7n	AGAP000714	X: 12,757,892-12,760,135 (-)
AgamGLUvir7s	AgIR7s	AGAP013409	X: 12,762,561-12,764,534 (-)
AgamGLUvir7t	AgIR7t	AGAP002763	2R: 26,797,736-26,799,713 (+)
AgamGLUvir7u	AgIR7u	AGAP013285	2R: 26,795,525-26,797,440 (+)
AgamGLUvir7w	AgIR7w	AGAP013416	2R: 26,799,941-26,801,942 (+)
AgamGLUvir7x	AgIR7x	AGAP013520	X: 12,767,186-12,769,138 (+)
AgamGLUvir7y	AgIR7y	AGAP013172	X: 12,771,474-12,773,520 (+)
AgamGLUvir8a	AgIR8a	AGAP010411	3L: 2,867,389-2,870,222 (-)
AgamGLUvir21a	AgIR21a	AGAP008511	3R: 11,468,382-11,471,590 (-)
AgamGLUvir25a	AgIR25a	AGAP010272	3R: 51,917,004-51,920,704 (-)
AgamGLUvir31a	AgIR31a	AGAP009014	3R: 23,550,606-23,552,617 (-)
AgamGLUvir40a	AgIR40a	AGAP004021	2R: 47,922,057-47,924,755 (+)
AgamGLUvir41a	AgIR41a	AGAP002904	2R: 29,130,386-29,133,257 (-)
AgamGLUvir41b	AgIR41b	AGAP008759	3R: 17,137,047-17,139,240 (+)
AgamGLUvir41c	AgIR41c	AGAP012951	2R: 39,202,960-3',205,188 (-)
AgamGLUvir41n	AgIR41n	AGAP003531	2R: 39,207,749-3',210,030 (-)
AgamGLUvir41t.1	AgIR41t.1	AGAP004432	2R: 55,960,058-55,962,321 (+)
AgamGLUvir41t.2	AgIR41t.2	AGAP012969	2R: 55,988,209-55,990,472 (+)
AgamGLUvir60a	AgIR60a	AGAP011943	3L: 35,436,382-35,438,481 (-)
AgamGLUvir64a	AgIR64a	AGAP004923	2L: 6,087,139-6,107,636 (-)
AgamGLUvir68a	AgIR68a	AGAP007951	3R: 3,275,939-3,278,326 (+)
AgamGLUvir75d	AgIR75d	AGAP004969	2L: 7,301,252-7,303,528 (+)
AgamGLUvir75g	AgIR75g	AGAP013085	2R: 10,846,334-10,848,911 (-)
AgamGLUvir75h.1	AgIR75h.1	AGAP001811	2R: 10,851,283-10,853,577 (+)
AgamGLUvir75h.2	AgIR75h.2	AGAP001812	2R: 10,855,568-10,857,858 (+)
AgamGLUvir75k	AgIR75k	AGAP007498	2L: 46,923,249-46,925,420 (+)
AgamGLUvir75l	AgIR75l	AGAP005466	2L: 15,949,821-15,952,169 (-)
AgamGLUvir76b	AgIR76b	AGAP011968	3L: 35,687,053-35,692,621 (-)
AgamGLUvIR93a	AgIR93a	AGAP000256	X: 4,900,441-4,905,540 (-)
AgamGLUvir100a	AgIR100a	AGAP000140	X: 2,149,577-2,152,578 (+)
AgamGLUvir100h	AgIR100h	AGAP000293	X: 5,199,638-5,204,600 (+)
AgamGLUvir100i	AgIR100i	AGAP004475	2R: 56,749,587-56,751,832 (+)
AgamGLUvir101	AgIR101	AGAP013425	2R: 42,015,346-42,017,474 (+)
AgamGLUvir133	AgIR133	AGAP005677	2L: 18,651,360-18,653,143 (-)
AgamGLUvir134	AgIR134	AGAP005678	2L: 18,657,551-18,659,242 (-)
AgamGLUvir135	AgIR135	AGAP005679	2L: 18,662,281-18,664,071 (-)
AgamGLUvir136	AgIR136	AGAP006440	2L: 31,959,226-31,960,962 (-)
AgamGLUvir137	AgIR137	-	2L: 31,957,279-31,958,959 (-)
AgamGLUvir138	AgIR138	-	2L: 31,954,985-31,956,676 (-)
AgamGLUvir139	AgIR139	AGAP006691	2L: 36,737,099-36,738,805 (-)
AgamGLUvir140.1	AgIR140.1	AGAP013242	2R: 16,645,150-16,647,020 (+)
AgamGLUvir140.2	AgIR140.2	AGAP013436	2R: 16,642,268-16,643,951 (+)
AgamGLUvir141	AgIR141	AGAP013473	2R: 31,458,247-31,463,898 (+)
AgamGLUvir142	AgIR142	AGAP006407	2L: 31,561,183-31,564,566 (+)

Table 1. Annotation of AgIR family members. Nomenclature, and chromosome positions 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). AgGLURI and AgGLURIIb represent partial peptides where the 5' end of the gene has not been annotated.

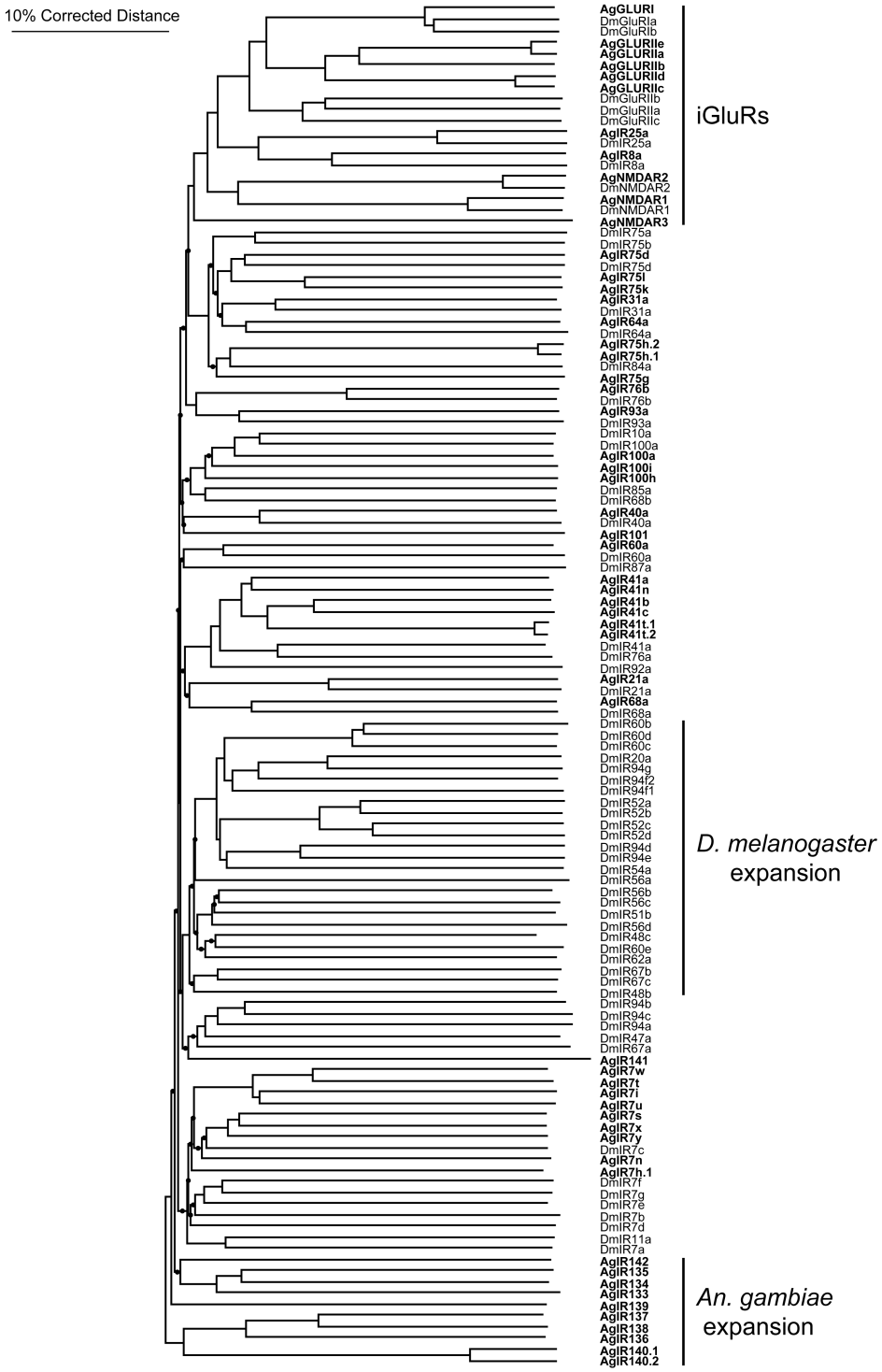

10% Corrected Distance
AgGLURI
DmGluRIa
DmGluRIb
AgGLURIIe
AgGLURIIa
AgGLURIIb
AgGLURIId
AgGLURIIc
DmGluRIIb
DmGluRIIa
DmGluRIIc
AgIR25a
DmIR25a
AgIR8a
DmIR8a
AgNMDAR2
DmNMDAR2
AgNMDAR1
DmNMDAR1
AgNMDAR3
DmIR75a
DmIR75b
AgIR75d
DmIR75d
AgIR75l
AgIR75k
AgIR31a
DmIR31a
AgIR64a
DmIR64a
AgIR75h.2
AgIR75h.1
DmIR84a
AgIR75g
AgIR76b
DmIR76b
AgIR93a
DmIR93a
DmIR10a
DmIR100a
AgIR100a
AgIR100i
AgIR100h
DmIR85a
DmIR68b
AgIR40a
DmIR40a
AgIR101
AgIR60a
DmIR60a
DmIR87a
AgIR41a
AgIR41n
AgIR41b
AgIR41c
AgIR41t.1
AgIR41t.2
DmIR41a
DmIR76a
DmIR92a
AgIR21a
DmIR21a
AgIR68a
DmIR68a
DmIR60b
DmIR60d
DmIR60c
DmIR20a
DmIR94g
DmIR94f2
DmIR94f1
DmIR52a
DmIR52b
DmIR52c
DmIR52d
DmIR94d
DmIR94e
DmIR54a
DmIR56a
DmIR56b
DmIR56c
DmIR51b
DmIR56d
DmIR48c
DmIR60e
DmIR62a
DmIR67b
DmIR67c
DmIR48b
DmIR94b
DmIR94c
DmIR94a
DmIR47a
DmIR67a
AgIR141
AgIR7w
AgIR7t
AgIR7i
AgIR7u
AgIR7s
AgIR7x
AgIR7y
DmIR7c
AgIR7n
AgIR7h.1
DmIR7f
DmIR7g
DmIR7e
DmIR7b
DmIR7d
DmIR11a
DmIR7a
AgIR142
AgIR135
AgIR134
AgIR133
AgIR139
AgIR137
AgIR138
AgIR136
AgIR140.1
AgIR140.2
iGluRs
D. melanogaster expansion
An. gambiae expansion

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%.

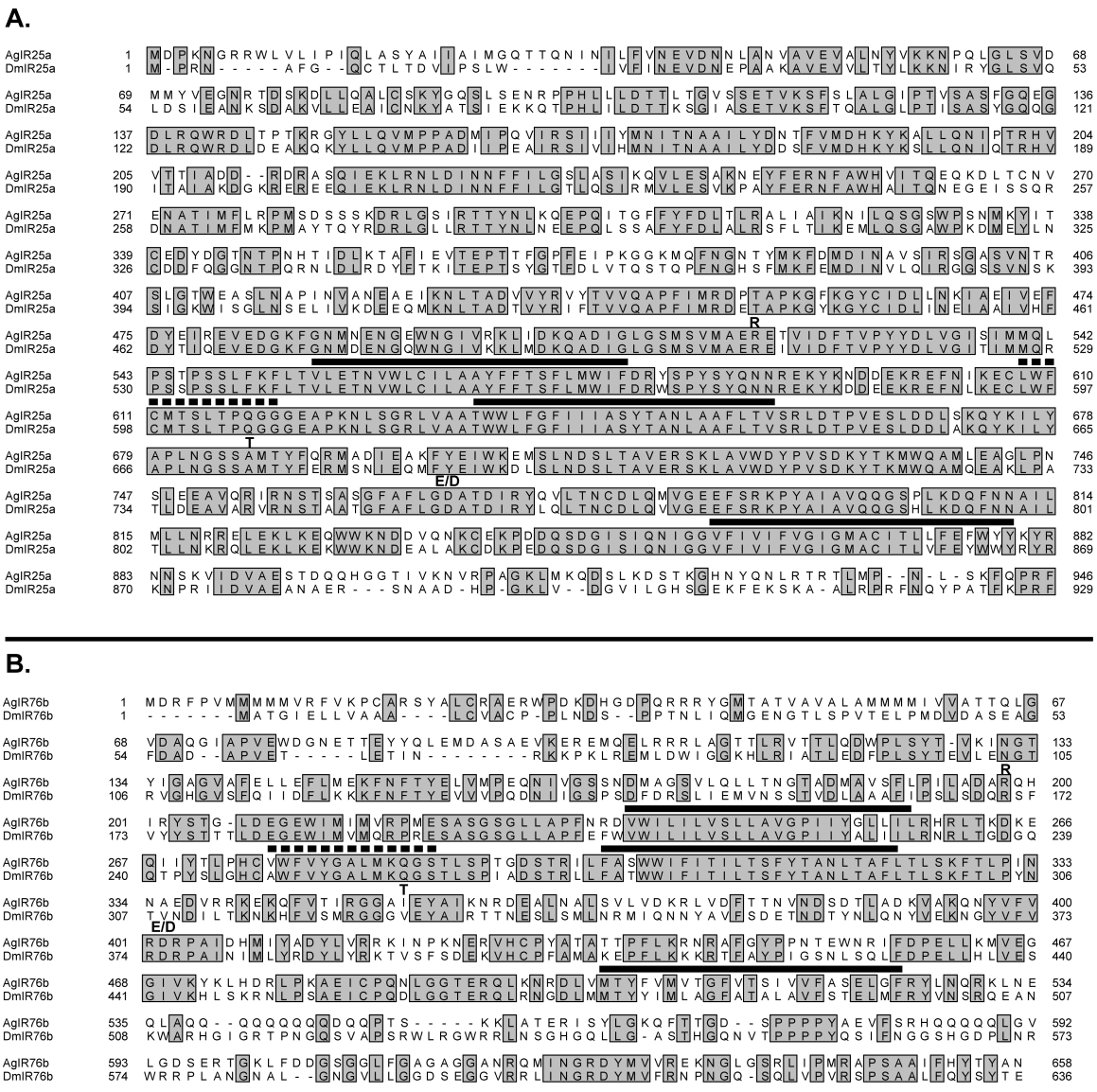


Figure 10. Representative alignments of AgIR and DmIR homologs. (A) IR25a peptide alignment. (B) IR76b peptide alignment. Amino acid sequences (single letter code) were aligned using ClustalX. Identical residues are shaded. Bold lines above residues indicate predicted transmembrane helices, while the 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.

probe in antennal sections revealed broad expression in antennal cells that are likely to be olfactory receptor neurons (Figure 12; note: Table 2 and Figure 12 added after publication).

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 studies (Xia et al. 2008). The first was 3-methylphenol (3MP), which was shown to activate AgOR-dependent pathways and evoke robust behavioral responses in larvae (Xia et al. 2008). 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 13). Furthermore, larval responses to 10-4 dilutions of 3MP were significantly altered in larvae injected with *AgOr7* siRNA, whereas control or buffer-injected larval responses were statistically equivalent to uninjected control larvae (Figures 14 and 15A). AgOR40 is one of 3 larval AgORs with a demonstrated sensitivity to 3MP (Xia et al. 2008). 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.

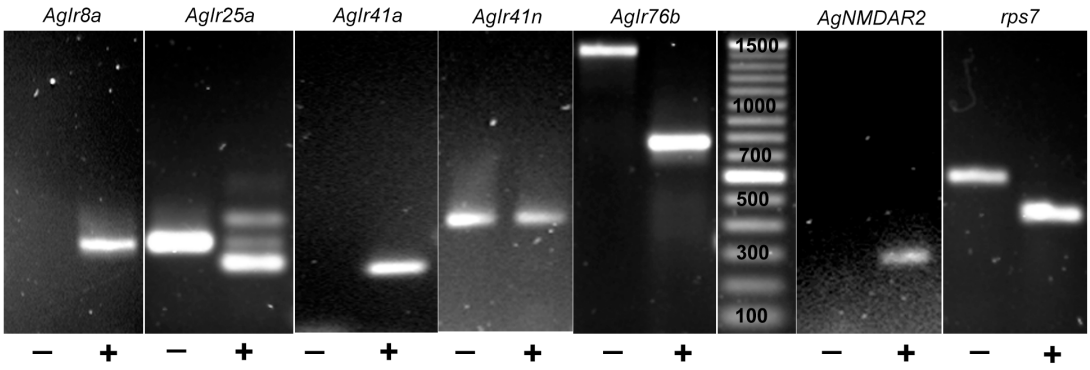


Figure 11. Expression of *AgIrs* in larval antennae. Composite image of agarose gel lanes showing cDNA (lower) and gDNA (upper) bands following RT-PCR using *AgIr*-specific primers as indicated above lanes. Minus (2) and plus (+) signs below lanes indicate the presence or absence of reverse transcriptase in first strand cDNA synthesis reaction, respectively. Bands (base pairs): *AgIr8a* cDNA (319); *AgIr25a* cDNA (271), gDNA (334); *AgIr41a* cDNA (245); *AgIr41n* cDNA (336, not present), gDNA (417); *AgNMDAR2* cDNA (328); *AgIr76b* cDNA (770), gDNA (1414); *rps7* cDNA (460), gDNA (609). No genomic bands were expected for *AgIr8a*, *AgIr41a*, 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).

gene	fa RPKM	ma RPKM	fp RPKM	mp RPKM
Aglr7h.1	0.14	0.03	0.03	0.09
Aglr7i	**5.85**	0.24	**1.05**	**2.35**
Aglr7n	0.76	0.76	0.12	0.16
Aglr7s	0.52	0.11	0.20	0.07
Aglr7t	**13.55**	**3.54**	**1.89**	**2.35**
Aglr7u	**7.29**	0.92	**1.03**	**1.85**
Aglr7w	**23.63**	**8.78**	**4.76**	**4.68**
Aglr7x	0.14	0.00	0.00	0.07
Aglr7y	0.00	0.00	0.00	0.05
Aglr8a	**49.04**	**8.90**	0.71	0.55
Aglr21a	**14.84**	**5.54**	0.14	0.22
Aglr25a	**121.59**	**41.08**	**55.81**	**9.55**
Aglr31a	**38.18**	**2.76**	0.13	0.20
Aglr40a	**1.69**	0.54	0.00	0.06
Aglr41a	**16.20**	**3.61**	**2.67**	0.58
Aglr41b	**5.53**	**1.50**	0.39	0.60
Aglr41c	**62.07**	**6.86**	**2.53**	**1.56**
Aglr41n	**65.11**	**6.04**	**3.67**	**2.25**
Aglr41t.1	**9.02**	**3.63**	0.85	**1.25**
Aglr41t.2	**33.18**	**5.57**	0.74	**1.26**
Aglr60a	0.00	0.06	0.00	0.00
Aglr64a	**41.41**	**6.31**	**1.09**	**3.21**
Aglr68a	0.45	0.23	0.00	0.04
Aglr75d	**10.61**	**4.82**	**1.18**	0.25
Aglr75g	**15.30**	**13.28**	0.38	0.51
Aglr75h.1	**3.50**	**1.83**	0.00	0.00
Aglr75h.2	**33.39**	**3.85**	0.44	0.43
Aglr75k	**21.63**	**5.87**	**4.77**	**2.51**
Aglr75l	**79.07**	**9.77**	0.24	0.28
Aglr76b	**167.67**	**58.34**	**13.11**	**12.32**
Aglr93a	**49.00**	**6.47**	0.31	0.47
Aglr100a	**19.62**	**3.95**	**13.13**	**2.96**
Aglr100h	**1.58**	**1.08**	**1.32**	**1.27**
Aglr100i	**2.83**	0.53	0.15	0.43
Aglr101	0.44	0.16	0.42	**3.35**
Aglr133	0.33	0.73	**1.03**	0.51
Aglr134	0.12	0.32	0.58	0.24
Aglr135	0.16	0.19	0.38	0.33
Aglr136	0.04	0.02	0.26	0.01
Aglr139	0.04	0.00	0.00	0.00
Aglr140.1	0.02	0.00	0.04	0.02
Aglr140.2	0.02	0.07	0.00	0.03
Aglr141	0.00	0.12	0.00	0.05
Aglr142	0.15	0.58	0.10	0.03

Table 2 Expression of *Aglr* family members in antennae and maxillary palps. Columns: *Aglr* gene names; Normalized Expression Values in antennae and maxillary palps. fa – female antenna, ma – male antenna, fp – female palp, mp – male palp. RPKM – Reads Per Kilobase per Million reads (see Methods, Chapter III for explanation). RPKM values <1.0 are shown in gray type, RPKM values >1.0 are shown in boldface type and green highlight.

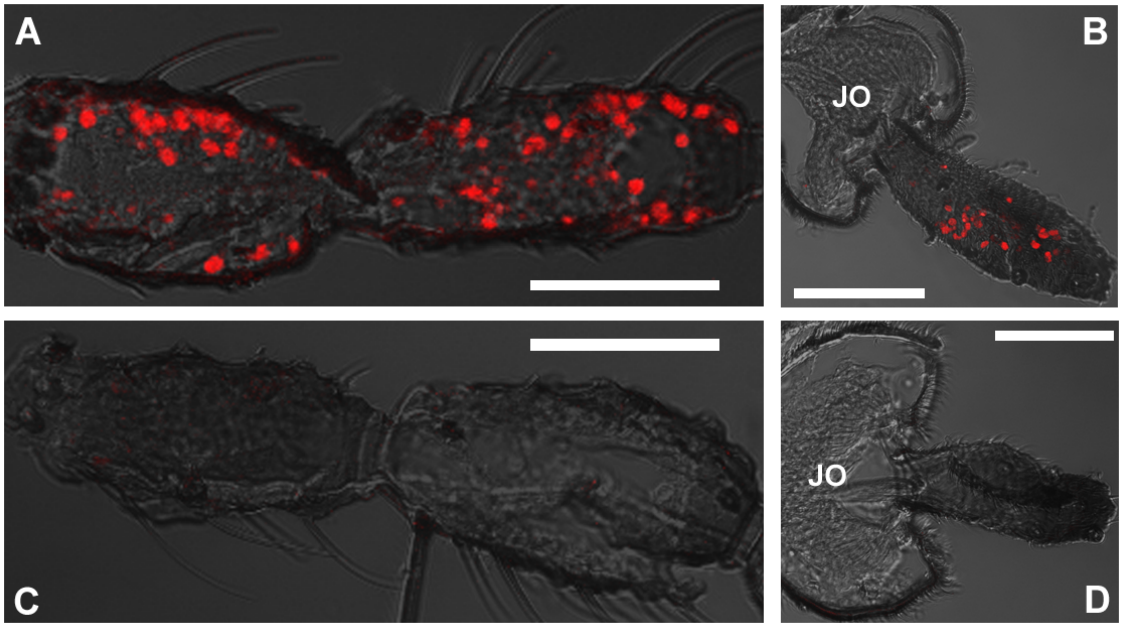


Figure 12. Expression of *AgIr25a* in adult female antennae. (A) Dig-antisense probe (with anti-dig Cy3) showing labeling of cells in antennal flagellomeres 4-5. (B) Dig-antisense probe (with anti-dig Cy3) showing labeling of cells in antennal flagellomere 1. (C,D) Dig-sense probe (with anti-dig Cy3) showing low background labeling in flagellomeres 4-5 and 1, respectively. JO: Johnston's organ. Scale bars are 50μm.

The next set of studies employed butylamine, a unitary odorant that has been shown to activate grooved-peg ORNs in *An. gambiae* (Qiu et al. 2006) and *Culex quinquefasciatus* mosquitoes (Syed et al. 2009). As was the case for 3MP, uninjected *An. gambiae* larvae displayed robust dose-dependent responses to butylamine (Figure 13). In contrast to the *AgOr7*-dependent nature of larval responses to 3MP, larval responses to butylamine were indistinguishable among animals treated with *AgOr7* and control siRNAs or microinjected with buffer alone (Figures 14 and 15B). Based on their homology to DmIRs, which have been shown to mediate responses to amines and other odorants in *Drosophila* (Benton et al. 2009), 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—*AgIr76b*—displayed siRNA-specific effects on larval responses to butylamine. Microinjection of *AgIr76b* siRNAs reduced *AgIr76b* mRNA levels (Figure 16) and led to significant alterations in larval responses to butylamine (Figures 14 and 15B). Larval responses to butylamine were unaffected in *AgOr7* knockdowns and by microinjection of non-specific siRNAs or buffer-alone controls (Figures 14 and 15B).

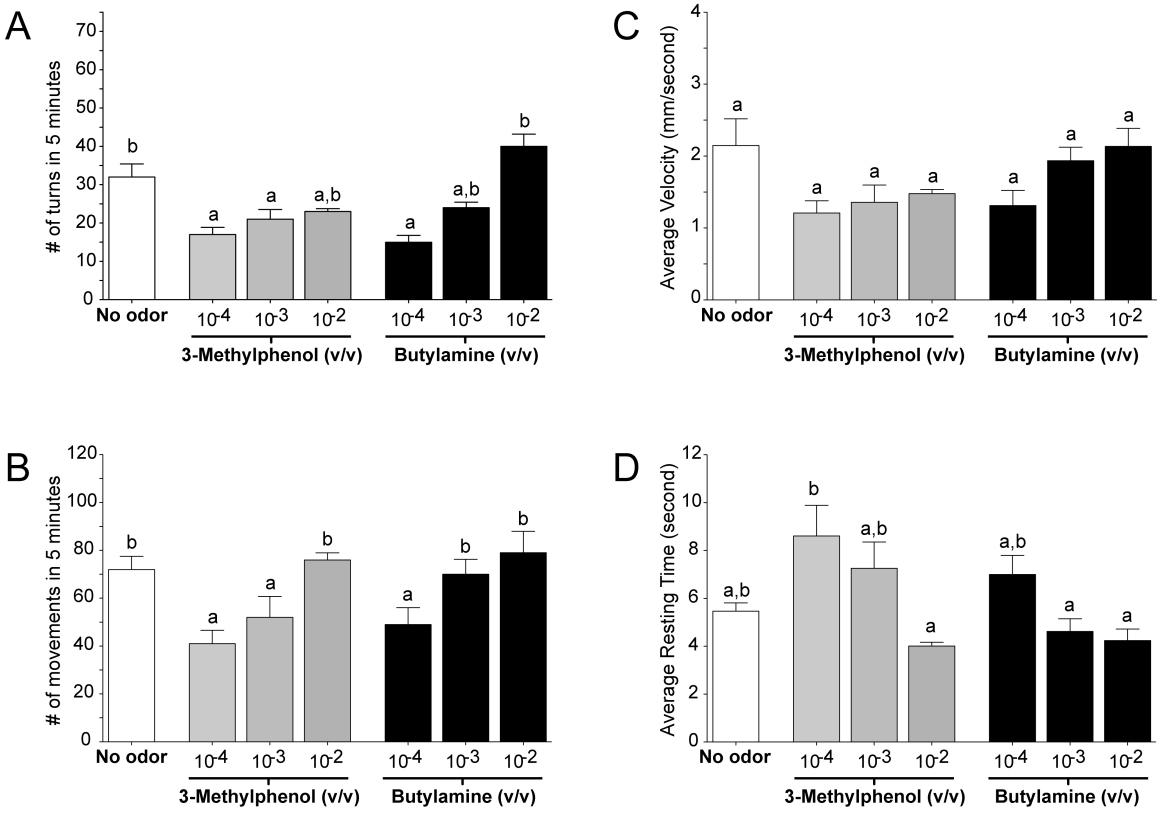


Figure 13. Behavioral effects of 3MP and Butylamine on *An. gambiae*. Larval responses to increasing dilutions (v/v) of 3MP and butylamine are displayed: total number of turns/assay (A), average number of movements/assay (B), average velocity (C), and resting time (D). With the exception of average velocity, 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 +/- SE, n = 10.

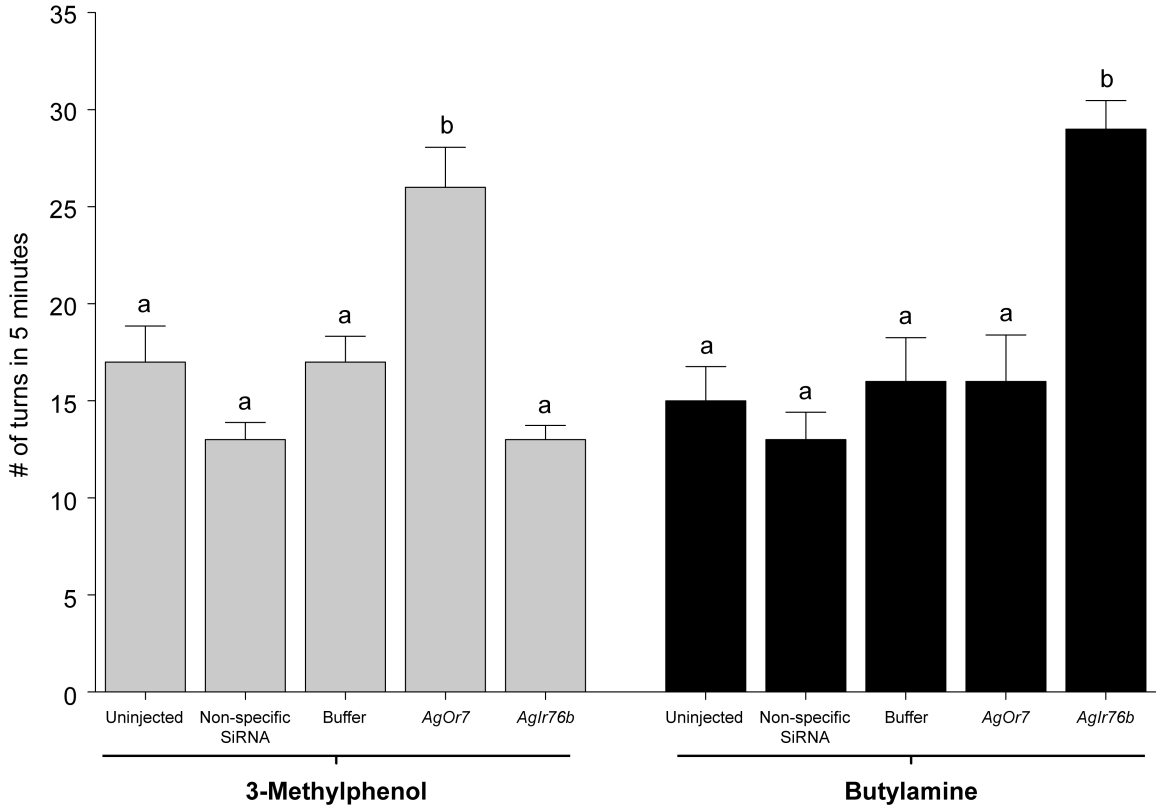


Figure 14. Olfactory responses to 3-methylphenol and butylamine are mediated by distinct signaling pathways. The turning rates exhibited by uninjected larvae as well as those receiving mock (buffer-alone), non-specific, or siRNA injections in response to 10-4 v/v dilutions of 3-methylphenol or butylamine were assessed independently over a 5 min time lapse. (A) Larval responses to 3-methylphenol were significantly altered by *AgOr7* knockdown but unaffected by *AgIr76b* silencing. (B) Conversely, responses to butylamine were sensitive to reduction in *AgIr76b* mRNA levels but indifferent to silencing of *AgOr7* expression.

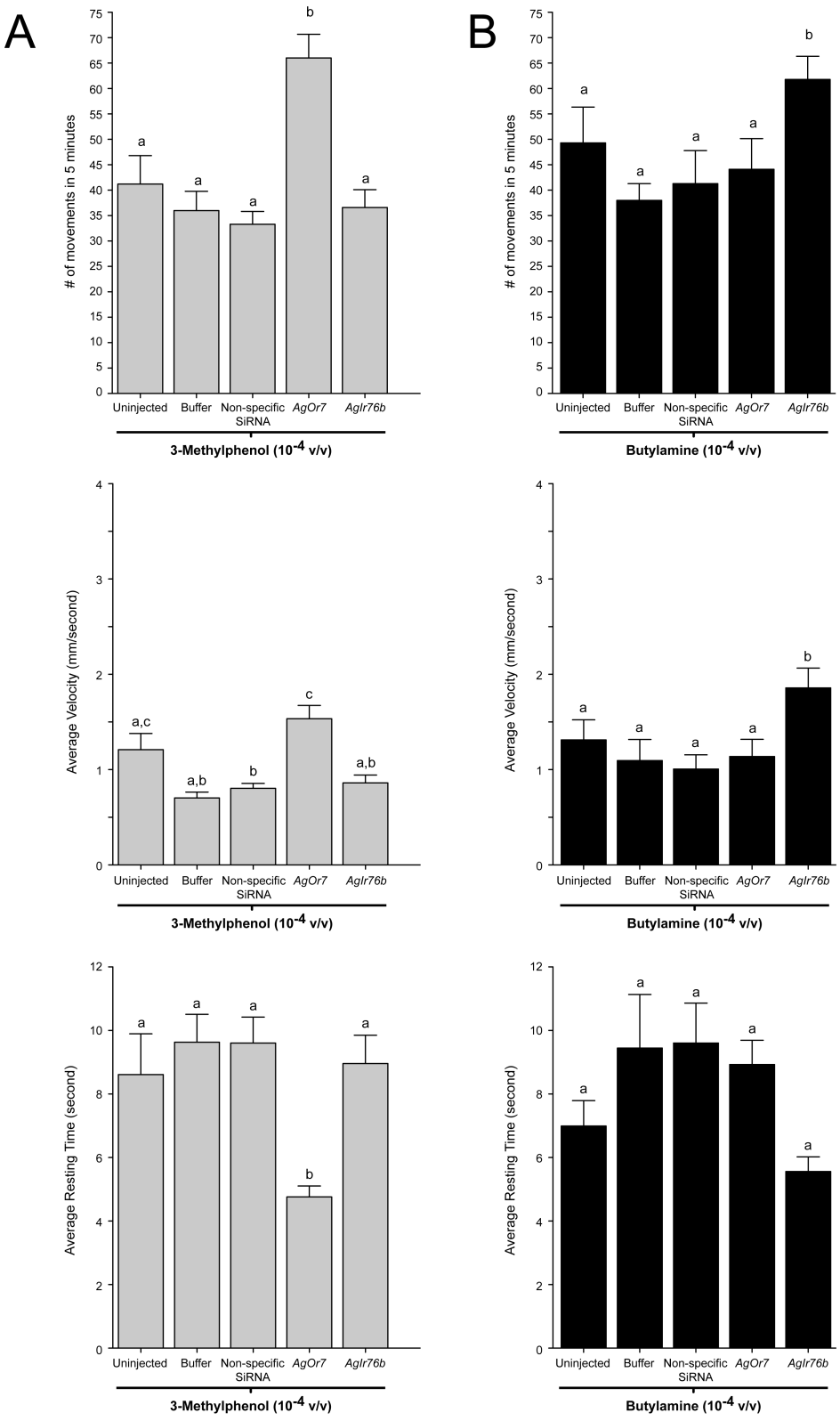

A
B
# of movements in 5 minutes
Average Velocity (mm/second)
Average Resting Time (second)
Uninjected
Buffer
Non-specific SiRNA
AgOr7
AgIr76b
3-Methylphenol (10-4 v/v)
Butylamine (10-4 v/v)

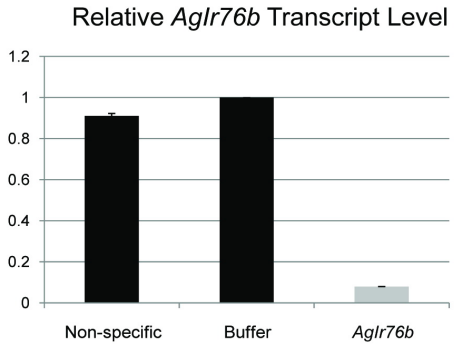
Figure 15. Odorant-specific differential effects of *AgOr/AgIr* knockdown. Averaged responses of buffer, non-specific, *AgOr7*, and *AgIr76b* siRNA injected larvae in the presence of 10-4 v/v dilutions of 3-methylphenol (3MP, left panels) or butylamine (BA, right panels). Histograms of larval movement (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 +/- SE, n = 10.

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.



Treatment	*AgIr76b* mRNA level fold change		
Non-specific siRNA inject	0.87	0.90	0.95
Buffer inject	1	1	1
AgIr76b siRNA inject	1/17.5	1/14.6	1/8.9

Gene	Treatment	Cycle Threshold (CT) Value		
Rps7 Primer Effi. = 2	Non-specific siRNA injected	17.74	17.82	17.85
	AgIr76b siRNA injected	17.42	17.56	17.80
	Buffer injected	17.84	18.05	17.98
AgIr76b Primer Effi. = 1.995	Non-specific siRNA injected	29.49	29.97	30.01
	AgIr76b siRNA injected	33.09	33.43	33.06
	Buffer injected	29.39	30.04	30.07

Figure 16 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 µg) 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 (2001). *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.

Previous studies (Ditzen et al. 2008) 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* (Xia et al. 2008) that showed robust DEET-mediated behavioral responses that correlated with a discrete population of larval ORNs co-expressing AgOR7/AgOR40 as well as specific DEET stimulation of *Xenopus* oocytes injected with AgOR7/AgOR40 cRNAs. This hypothesis is also supported by other studies that describe DEET-mediated activation of a subset of ORNs in Culex mosquitoes (Syed et al. 2008) and more recent work in *Aedes aeqypti* suggesting that DEET sensitivity is a genetically determined characteristic affecting the functionality of discrete ORNs (Stanczyk et al. 2010). While the reduction in DEET-mediated repellent responses in larvae undergoing RNAi mediated silencing of *AgOr7* 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 *Or83b* family member *AgOr7*, 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 *AgOr7* transcripts (Figures 6, 14). 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 *AgIRs8a* 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 (Carey et al. 2010; Wang et al. 2010) 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 (Cork and Park 1996; Bernier

et al. 2000). These groups of odorants are thought to play essential roles in host-seeking, oviposition, and other behaviors that are critical for Anopheline life cycles (Meijerink et al. 2000). Coincidently, 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 (Carey et al. 2010; Wang et al. 2010). 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 12) 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 *AgOr7* 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 (Carey et al. 2010; Wang et al. 2010), 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 sensilla (Meijerink et al. 2001)

that are devoid of AgOR7 (Pitts et al. 2004). Indeed, we have observed expression of multiple *AgIrs* in adult olfactory appendages (Table 2), supporting the hypothesis that this family of genes is involved in chemosensory signaling in adults (see Chapter III).

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.

References

Benton R, Sachse S, Michnick SW, and Vosshall LB (2006) Atypical membrane topology and heteromeric function of *Drosophila* odorant receptors in vivo. *PLoS Biol* 4: e20.

Benton R, Vannice KS, Gomez-Diaz C, and Vosshall LB (2009) Variant ionotropic glutamate receptors as chemosensory receptors in *Drosophila*. *Cell* 136: 149–162.

Bernier UR, Kline DL, Barnard DR, Schreck CE, and Yost RA (2000) Analysis of human skin emanations by gas chromatography/mass spectrometry. 2. Identification of volatile compounds that are candidate attractants for the yellow fever mosquito (*Aedes aegypti*). *Anal Chem* 72: 747–756.

Carey AF, Wang G, Su CY, Zwiebel LJ, and Carlson JR (2010) Odorant reception in the malaria mosquito *Anopheles gambiae*. *Nature* 464: 66–71.

Cork A and Park KC (1996) Identification of electrophysiologically-active compounds for the malaria mosquito, *Anopheles gambiae*, in human sweat extracts. *Med Vet Entomol* 10: 269–276.

Croset V, Rytz R, Cummins SF, Budd A, Brawand D, Kaessmann H, Gibson TJ, and Benton R (2010) Ancient protostome origin of chemosensory ionotropic glutamate receptors and the evolution of insect taste and olfaction. *PLoS Genet* 6: e1001064. doi:10.1371/journal.pgen.1001064.

Ditzen M, Pellegrino M, and Vosshall LB (2008) Insect odorant receptors are molecular targets of the insect repellent DEET. *Science* 319: 1838–1842.

Fox AN, Pitts RJ, Robertson HM, Carlson JR, and Zwiebel LJ (2001) Candidate odorant receptors from the malaria vector mosquito *Anopheles gambiae* and evidence of down-regulation in response to blood feeding. *Proc Natl Acad Sci U S A* 98: 14693–14697.

Hallem E, Ho MG, and Carlson JR (2004) The molecular basis of odor coding in the *Drosophila* antenna. *Cell* 117: 965–979.

Hill CA, Fox AN, Pitts RJ, Kent LB, Tan PL, et al. (2002) G protein-coupled receptors in *Anopheles gambiae*. *Science* 298: 176–178.

Kwon HW, Lu T, Rutzler M, and Zwiebel LJ (2006) Olfactory responses in a gustatory organ of the malaria vector mosquito *Anopheles gambiae*. *Proc Natl Acad Sci U S A* 103: 13526–13531.

Larsson MC, Domingos AI, Jones WD, Chiappe ME, Amrein H, et al. (2004) *Or83b* encodes a broadly expressed odorant receptor essential for *Drosophila* olfaction. *Neuron* 43: 703–714.

Lundin C, Kall L, Kreher SA, Kapp K, Sonnhammer EL, et al. (2007) Membrane topology of the *Drosophila OR83b* odorant receptor. *FEBS Lett* 581: 5601–5604.

Mayer ML (2006) Glutamate receptors at atomic resolution. *Nature* 440: 456–462.

Meijerink J, Braks MA, Braak AA, Adam W, Dekker T, et al. (2000) Identification of olfactory stimulants for *Anopheles gambiae* from human sweat samples. *J Chem Ecol* 26: 1367–1382.

Meijerink J, Braks MAH, and van Loon JJA (2001) Olfactory receptors on the antennae of the malaria mosquito *Anopheles gambiae* are sensitive to ammonia and other sweat-borne components. *J Insect Physiol* 47: 455–464.

Merrill CE, Pitts RJ, and Zwiebel LJ (2003) Molecular characterization of arrestin family members in the malaria vector mosquito, *Anopheles gambiae*. *Insect Molecular Biology* 12: 641–650.

Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* 29: e45.

Pitts RJ, Fox AN, and Zwiebel LJ (2004) A highly conserved candidate chemoreceptor expressed in both olfactory and gustatory tissues in the malaria vector *Anopheles gambiae*. *Proc Natl Acad Sci U S A* 101: 5058–5063.

Qiu YT, van Loon JJ, Takken W, Meijerink J, and Smid HM (2006) Olfactory coding in antennal neurons of the malaria mosquito, *Anopheles gambiae*. *Chem Senses* 31: 845–863.

Saitou N and Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* 4: 406–425.

Sato K, Pellegrino M, Nakagawa T, Nakagawa T, Vosshall LB, et al. (2008) Insect olfactory receptors are heteromeric ligand-gated ion channels. *Nature* 452: 1002–1006.

Stanczyk NM, Brookfield JF, Ignell R, Logan JG, and Field LM (2010) Behavioral insensitivity to DEET in *Aedes aegypti* is a genetically determined trait residing in changes in sensillum function. *Proc Natl Acad Sci U S A* 107: 8575–8580.

Syed Z and Leal WS (2008) Mosquitoes smell and avoid the insect repellent DEET. *Proc Natl Acad Sci U S A.*

Syed Z and Leal WS (2009) Acute olfactory response of Culex mosquitoes to a human- and bird-derived attractant. *Proc Natl Acad Sci* U S A 106: 18803–18808.

Takken W and Knols BG (1999) Odor-mediated behavior of Afrotropical malaria mosquitoes. *Annu Rev Entomol* 44: 131–157.

Wang G, Carey AF, Carlson JR, and Zwiebel LJ (2010) Molecular basis of odor coding in the malaria vector mosquito *Anopheles gambiae*. *Proc Natl Acad Sci U S A* 107: 4418-23.

Wicher D, Schafer R, Bauernfeind R, Stensmyr MC, Heller R, et al. (2008) *Drosophila* odorant receptors are both ligand-gated and cyclic-nucleotide-activated cation channels. *Nature* 452: 1007–11.

Xia Y, Wang G, Buscariollo D, Pitts JR, Wenger H, and Zwiebel LJ (2008) The molecular basis of olfactory-based behavior in *Anopheles gambiae* larvae. *Proc Natl Acad Sci U S A* 105: 6433–38.

CHAPTER III

TRANSCRIPTOME PROFILING OF CHEMOSENSORY APPENDAGES IN THE MALARIA VECTOR *ANOPHELES GAMBIAE* REVEALS TISSUE- AND SEX-SPECIFIC SIGNATURES OF ODOR CODING

Preface

The following manuscript by Pitts et al. was submitted to *BMC Genomics* on 3/24/11 and is currently under review (4/1/11). My contribution to this work included experimental design, mosquito tissue dissections, data analysis, figure preparation, and manuscript writing.

Introduction

Insects rely heavily upon chemosensation, the ability to detect and react to environmental chemical cues, in virtually every aspect of their life cycle (Gillott 2005). Chemosensation is critical to food source identification, predator avoidance, oviposition site selection, kin recognition, mate choice, and toxic compound avoidance. In insects, chemosensory neurons are contained within distinct tissues on many parts of the body, most conspicuously on the antennae and the maxillary palps located on the head. These appendages are decorated with sensory hairs, or sensilla, that house the neurons in which families of insect-specific receptors and other proteins transduce chemosensory signals (for reviews

see Sato and Touhara 2009; de Bruyne and Baker 2008; Gilliott 2005; McIver 1982). Some insect sensory neurons have become highly specialized for the detection of single compounds, while others function more generally and are sensitive to multiple compounds (Kaissling 2009; Touhara and Vosshall 2009; Hallem et al., 2006). While the physiological and cellular basis of insect chemosensation has been studied for many years, its molecular underpinnings have only recently begun to be elucidated.

In mosquitoes, host-seeking behavior is driven largely by olfaction (Zwiebel and Takken 2004; Takken and Knols 1999). *An. gambiae* females display a strong preference for human hosts (anthropophily), which contributes substantially to their ability to transmit human diseases, including malaria (Zwiebel and Takken 2004; Takken and Knols 1999; Costantini et al., 1999). The identification of chemoreceptor gene families in the *An. gambiae* genome (Hill et al., 2002; Liu et al., 2010) has facilitated the correlation of behavioral observations and physiological sensitivities to receptor expression (Fox et al., 2001; Pitts et al., 2004; Kwon et al., 2006; Lu et al., 2007). Specific chemoreceptors expressed in antennal and palpal neurons of *An. gambiae* are sensitive to host odors, including volatile components produced from bacteria associated with human skin (Hallem et al., 2004; Carey et al., 2010; Wang et al 2010; Verhulst et al., 2010). As a consequence, the function of select chemoreceptor genes in *An. gambiae* has been linked to semiochemicals

that are integral to specific host seeking behaviors. Despite this progress, very little of the downstream signaling events and regulation of chemoreceptor function is known. Moreover, the potential chemosensory bases of sexually distinct behaviors in *An. gambiae* are poorly understood (Howell and Knols 2009; Zwiebel and Takken 2004; Clements 1999).

RNA-seq offers great potential to comprehensively study gene expression in head appendages of *An. gambiae* to provide insight into the molecular foundations of chemoreception. While several microarray-based studies have examined global transcript abundance in *An. gambiae* (Aguilar et al., 2010; Das et al., 2010; Cook and Sinkins 2010; Baton et al., 2009; Warr et al., 2007; Marinotti et al., 2006; Aguilar et al., 2005; Marinotti et al., 2005), none has focused exclusively on chemoreceptive tissues. Moreover, unlike microarrays and older methods, RNA-seq provides transcriptome-wide sequence coverage with unbiased, highly quantitative results (Wang, Gerstein, Snyder, 2009) and greatly improved sensitivity (‘t Hoen et al., 2008; Mortazavi et al., 2008). To date, RNA-seq has been used to address several functional and evolutionary questions pertaining to mosquito biology (Gibbons et al., 2009; Hittinger et al., 2010; Crawford JE et al., 2010; Neira-Oviedo 2010; Bonizzoni et al, 2011).

Here we have utilized RNA-seq to quantify global abundance levels of poly-adenylated transcripts of *An. gambiae* whole adults, antennae and maxillary palps across sexes. By mapping the generated short read sequences against the full set of annotated *An. gambiae* transcripts we

have generated six tissue- and sex-specific transcriptome profiles. As expected, gene families with well-established chemosensory function display antenna- or palp-enhanced abundance, with antennae showing enhancement of a larger number of these genes. We also have identified numerous members of other gene families that are enhanced in either antennae or maxillary palps, such as biotransformation enzymes, transcription factors, transmembrane receptors, ion channels, transporters and proteases which are likely to function in chemosensory pathways. Our data also revealed an unanticipated level of sexual monomorphism with respect to the abundance and distribution of known chemoreceptive functional classes in the antenna and the maxillary palp. Taken as a whole, this study greatly broadens our understanding of the molecular processes involved in peripheral sensory appendages, raising new questions about basic dipteran biology and offering the potential for novel targets for insect control.

Methods

Mosquito Rearing

An. gambiae sensu stricto, which originated from Suakoko, Liberia (della Torre et al 1996), were reared as described (Qiu et al 2004).

RNA Isolation and Sequencing

Tissues were hand dissected from 4-6 d.o. adult *An. gambiae* at ~ZT12 and immediately placed in RNA Later Ice (Ambion Corp.; Austin, TX) on ice. Total RNA was isolated from each sample using RNeasy columns (Qiagen Inc.; Carlsbad, CA) according to the manufacturer's protocol. mRNA isolation and cDNA library preparation were carried out using the Illumina mRNA sequencing kit (Illumina Inc.; San Diego, CA). Libraries were sequenced using an Illumina Genome Analyzer II.

AgOr and AgObp Reannotations

Novel *AgOrs* were identified by tBLASTn searches (www.ncbi.org; default parameters) using the previously identified AgOR peptides as queries. Two new candidate *AgOrs* were identified and have been named *AgOrs 80* and *81*. Furthermore, *AgOrs 12*, *67*, *78* and *79* have been purged from the *AgOr* family as apparent duplication errors in the original assembly (Table 2). Three new candidate *AgObps* (*69*, *70* and *71*) were identified using similar tBLASTn searches and were added to the family based on two criteria: the candidate genes possessed motifs that exemplify the *Obp* family (Vogt 1981; Kruse 2003; Xu et al., 2003; Hekmat-Scafe et al., 2002) and each gene model encoded a unique transcript. Other genes resembling *Obps* were identified, but have not been included in the named members of the *AgObp* family. However we recognize the possibility that these genes may ultimately prove to be unique, or function as odor-carriers. These will be discussed in more detail

below. Similarly, *AgObps 16*, *17*, *24, 58, 59, 61, 61,* and 65 were purged from the *AgObp* family as apparent duplication errors in assembly. All modifications to the *AgOr* and *AgObp* families have been submitted to VectorBase.

Data Processing and Expression Profiling

Individual *Illumina* read files were mapped to the recently updated (Dec. 2010) soft Repeat Masked version of the assembled *An. gambiae* genome, to the mitochondrial genome, and to the annotated *An. gambiae* transcripts (www.VectorBase.org). For purposes of mapping, all alternate transcript isoforms for a given gene were condensed under that gene's respective AGAP designation. Prior to mapping, individual reads were quality checked and uniformly trimmed by 4 and 12 nucleotides on their 5- and 3-prime ends respectively to account for spurious adapter incorporation (5') and for sequencing reaction degeneration (3'). Mapping was carried out using seqmap software, configured to allow for a maximum of three mismatches per read. Processed mapping data was then consolidated based upon AGAP number and the results summarized by rseq. Abundance level output by rseq is reported in terms of unique reads, total weighted reads, and transcript length. Total weighted reads and AGAP transcript lengths were used to calculate a normalized abundance level of Reads per Kilobase per Million reads mapped (RPKMs), for every AGAP in every tissue type (Mortazavi et al., 2008).

PfamA Categorization

Peptide sequences from AgamP3.6 conceptual peptides (n=12,669) were compared to the PfamA dataset (The Pfam protein families database: Finn et al. 2010) using the default e-value threshold of 1.0.

Comparison of Tissue Expression Profiles

Statistical significance was assigned to each pairwise tissue comparison (antenna:body, palp:body, body:body) by setting up a Fisher's Exact test, evaluating gene-by-gene differences of weighted, mapped reads and total mapped reads for a given sample. The Agam3.6 transcript annotation contains 13319 unique, annotated transcripts and the statistical significance of the Fisher's Test was evaluated against a Bonferroni corrected p-value of 3.8x10-6.

AgOr RT-PCR Amplifications

Antennae from female and males, and male reproductive tract tissues (terminal abdominal segments) were hand dissected. RNA was isolated using a Trizol extraction as described (Lu et al. 2007). First strand cDNA was synthesized using the Transcriptor kit (Roche). PCR primers specific for *AgOrs* were as follows: *AgOr7*: TGCTGCTACACATGCTGAC and TAGGTGACAACGGCTCCAA; *AgOr35*:

TTCCTGTTCAACTGTGACTC and TATGAAGCCACCTTTGGTGA. PCR amplification conditions were: 92 degrees C for 1 min.; 35 cycles of (92C, 20s – 58C,20s – 72C,45s); 72C, 5min.

Results and Discussion

RNA Sequencing and Gene Mapping

As a means of inferring gene expression in chemosensory appendages we employed single-end short read (43bp) RNA-seq technology to characterize the relative abundances of poly-adenylated RNAs in antennae, maxillary palps and whole bodies of female and male adult mosquitoes. We established tissue- specific gene expression profiles for each of our six samples by mapping the read sequence files against the annotated *An. gambiae* transcriptome, using an approach that quantitated transcript abundance per gene and which accounted for all annotated transcripts per gene (see Materials and Methods). As our reference transcriptome, we used the AgamP3.6 version of the *An. gambiae* gene annotation, which contains 12,669 protein-coding genes and 650 non-coding RNAs (www.VectorBase.org). For each of the tissue types assayed, we obtained an average 30.5 million sequence reads per tissue type and mapped them to the *An. gambiae* transcriptome, nuclear and mitochondrial genomes (Table 1). On average, 57.4% of the reads

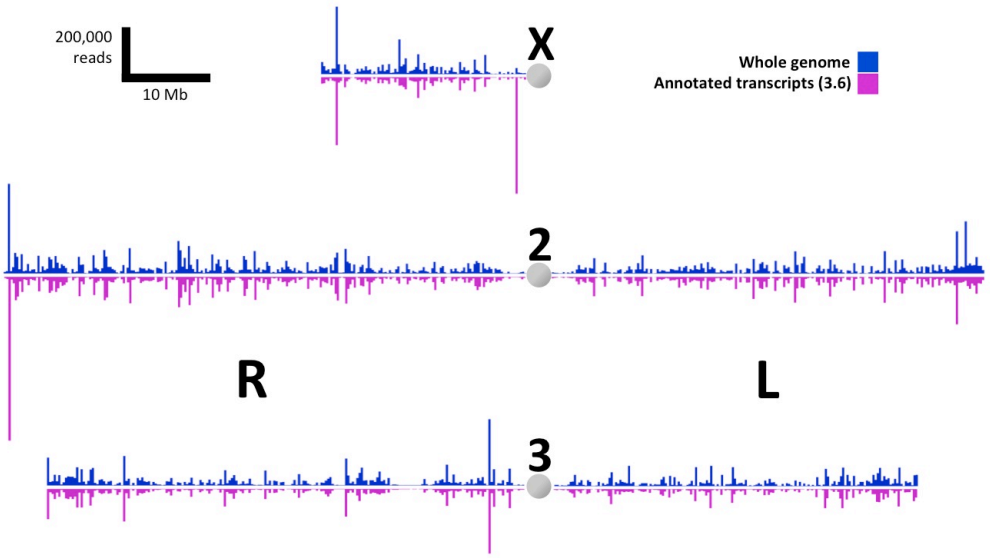


Figure 1. Read coverage of *An. gambiae* genome. Read count coverage of the nuclear genome (magenta) and of the transctiptome (blue). Vertical bars represent counts of sequence reads per 250kB interval along each of the three chromosomes.

per sample mapped to annotated genes, 91.5% to the nuclear genome (Table 1), and 2% to the mitochondrial genome (Table 1). Table S1 contains the complete RNA-seq data set described above, including the number of reads from each tissue sample that mapped to all 13,319 annotated *An. gambiae* genes.

On a whole-genome level, comparison of the density of reads sequenced from the female body along all chromosomes showed a high degree of correspondence between the number of reads mapped to the nuclear genome and the number of reads mapped to the transcriptome (Figure 1). That said, there are a few areas of asymmetry where a higher degree of mapping to either the transcriptome or to the genome was observed, most noticeably in the gene-rich autosomal telomeres and in several regions of the X chromosome (Figure 1). Greater mapping frequency to the transcriptome can generally be explained as reads that map to exon-exon junctions, which by their nature would not map to the genome. For example, the observed asymmetry in the 2R telomeric region is due to the high number of exon junction reads that mapped to two rhodopsin-family genes (Figure 1). Of the reads that mapped only to the genome, many of them are likely to represent unannotated 5'or 3' untranslated regions (UTRs). Moreover, there likely remain regions of the genome, most notably the Y-chromosome, where novel exons and transcripts remain (Li et al., 2006).

	Overall Totals		Weighted Mapped Read Counts			Gene Expression Summary			
tissue	reads	mapped reads (%)	transcriptome v3.6 (%)	nuc.	mito.	# genes	median RPKM	mean RPKM	std.dev. RPKM
FB	27.87M	25.36M (90.96)	16.61M (59.57)	14.68M	0.26M	12145	8.87	59.74	543.15
FA	25.98M	24.12M (92.85)	14.62M (56.26)	15.28M	0.08M	11722	9.38	59.22	732.65
FP	27.45M	25.98 (94.66)	15.29M (55.71)	16.70M	0.42M	12297	10.37	56.44	496.05
MB	31.88M	30.23M (94.82)	17.60M (55.22)	16.02M	2.41M	12253	8.34	54.01	424.05
MA	33.95M	32.14M (94.68)	18.23M (53.70)	21.43M	0.24M	11986	10.34	46.01	229.14
MP	35.71M	33.34M (93.37)	22.60M (63.29)	17.63M	0.54M	12146	8.40	49.14	286.49

Table 1. *An. gambiae* RNA-seq mapping and expression data. Cells in each row contain information corresponding to the tissue type listed. **Overall Totals:** reads - total number of short reads generated from each sample. mapped reads - the number (and percentage) of total reads that were mapped to the transcriptome, nuclear genome, and/or the mitochondrial genome. **Weighted Mapped Read Counts:** transcriptome v3.6 - the number (and percentage) of reads mapped to version 3.6 of the *An. gambiae* transcriptome. nuc. - the number the number of reads mapped to the assembled *An. gambiae* genome. mito. - the number of reads mapped to the *An. gambiae* mitochondrial genome. **Gene Expression Summary: #** genes - the total number of annotated genes in each tissue type having an RPKM greater than zero. median, mean, and std. deviation – calculated using RPKM values for each tissue type.

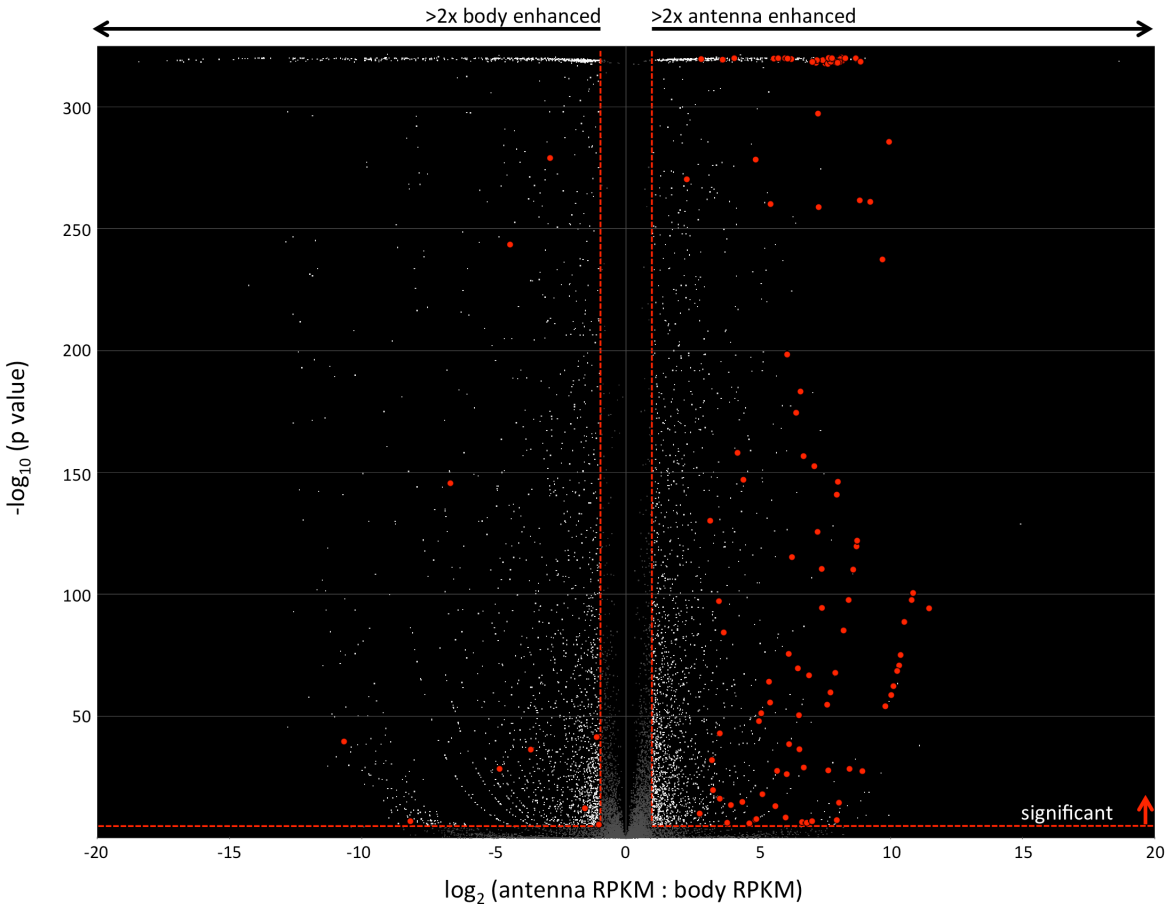


Figure 2. Gene expression in *An. gambiae* female antenna vs body. Volcano plot showing the relative abundance levels of genes in female whole body versus female antennae. The x-axis represents the $\log_2$ of the expression ratio (antenna RPKM: body RPKM) for each gene of the *An. gambiae* transcriptome. The y-axis represents the negative $\log_{10}$ of the p-value of Fisher's Exact test. White points (n=2201) represent genes that were both statistically significant (red horizontal line; p< 3.91e-06) and biologically significant (red vertical lines; greater than 2-fold difference in RPKMs). Gray points (n=10603) represent genes that either fell outside one or both of these significance criteria. Red points indicate the expression values of major chemosensory genes: *AgOrs*, *AgIrs*, *AgGrs*, and *AgObps*. RPKM values of 0.00 were transformed to 0.10 prior to calculating antenna:body ratios, such that those genes could also be represented on the plot.

To quantify relative differences in gene abundance levels, we calculated the **R**eads **P**er **K**ilobase per **M**illion (RPKM) reads value for each gene within a sample, a self-normalized value of absolute gene transcript abundance (Mortazavi et al., 2008). Mean and median RPKM values for each tissue type in this study were very similar across samples, as were the number of genes showing basal or greater levels of transcription (Table 1). RPKM values spanned more than 6 orders of magnitude for each of the tissue types examined.

We assessed fold-differences in transcript abundance by independently comparing ratios of RPKM values between pairs of tissues within each sex: antennae to bodies and maxillary palps to bodies. For each of these pairwise comparisons we performed a Fisher's Exact Test on counts of mapped reads to determine statistical significance using a Bonferroni-corrected p-value ($p < 3.9x10^{-6}$; see Materials and Methods). Furthermore, we use the term "enhanced" to describe any gene that displayed at least 2-fold significant difference in abundance between samples (Figure 2). These conservative criteria were applied to avoid false positives stemming from variations within the sample themselves, as well as to reduce the number of genes that was used for subsequent analyses (Robinson et al, 2010; Balweirz et al, 2009).

Gene Expression Profiling in Chemosensory Tissues

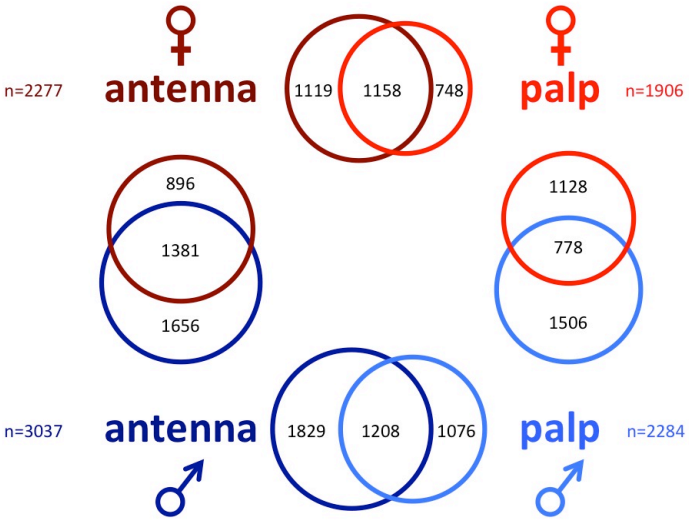
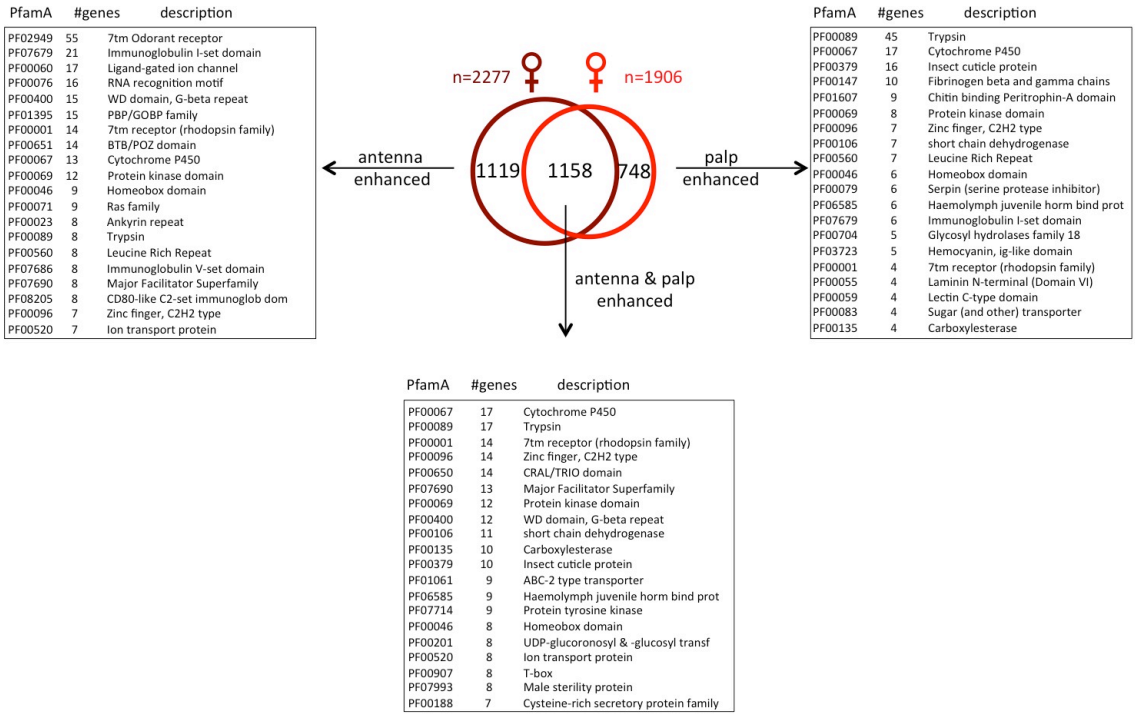


Figure 3: *An. gambiae* enhanced gene pairwise tissue comparisons. Proportional Venn diagrams showing the various pairwise comparisons made in this study. Overlap represents the subset of genes that are significantly enhanced in both tissues.

To examine global gene expression patterns, we compared RPKM values pairwise for whole bodies versus either antennae or maxillary palps in both sexes; one such comparison is shown in Figure 2. Specifically, 4,587 genes displayed directional enhancement in the female antenna to body comparison (Figure 2, white dots). Of those, 2,277 were enhanced in the antenna (Figure 2, right half). Similarly, we found that 1,906 genes were enhanced in female palps, 3,037 genes were enhanced in male antennae, and 2,284 genes were enhanced in male palps. These 4 gene sets formed the basis of our subsequent analyses where we compared enhanced gene profiles between chemosensory tissues and across sexes (Figure 3).

Comparing the enhanced gene sets between the female antennae and palps revealed significant overlap, with 1,158 genes (61% of palp set) enhanced in both tissues (Figure 4). Similarly, male antennae and palps showed significant overlap with 1,208 genes enhanced in both tissues (53% of palp set; Figure 5). Interestingly, the most well-represented gene families in both of these overlapping sets were 7-transmembrance receptors (PF00001), protein kinases (PF00069), cytochrome P450s (PF00067), trypsins (PF00089), carboxylesterases (PF00135), and potential transcription factors (PFs 00046 and 00096; Figures 4 and 5, bottom tables). However, we also observed several differentially enhanced gene sets between the antennae and palps (Figures 4 and 5). The *An.*



PfamA	#genes	description
PF02949	55	7tm Odorant receptor
PF07679	21	Immunoglobulin I-set domain
PF00060	17	Ligand-gated ion channel
PF00076	16	RNA recognition motif
PF00400	15	WD domain, G-beta repeat
PF01395	15	PBP/GOBP family
PF00001	14	7tm receptor (rhodopsin family)
PF00651	14	BTB/POZ domain
PF00067	13	Cytochrome P450
PF00069	12	Protein kinase domain
PF00046	9	Homeobox domain
PF00071	9	Ras family
PF00023	8	Ankyrin repeat
PF00089	8	Trypsin
PF00560	8	Leucine Rich Repeat
PF07686	8	Immunoglobulin V-set domain
PF07690	8	Major Facilitator Superfamily
PF08205	8	CD80-like C2-set immunoglob dom
PF00096	7	Zinc finger, C2H2 type
PF00520	7	Ion transport protein

PfamA	#genes	description
PF00089	45	Trypsin
PF00067	17	Cytochrome P450
PF00379	16	Insect cuticle protein
PF00147	10	Fibrinogen beta and gamma chains
PF01607	9	Chitin binding Peritrophin-A domain
PF00069	8	Protein kinase domain
PF00096	7	Zinc finger, C2H2 type
PF00106	7	short chain dehydrogenase
PF00560	7	Leucine Rich Repeat
PF00046	6	Homeobox domain
PF00079	6	Serpin (serine protease inhibitor)
PF06585	6	Haemolymph juvenile horm bind prot
PF07679	6	Immunoglobulin I-set domain
PF00704	5	Glycosyl hydrolases family 18
PF03723	5	Hemocyanin, ig-like domain
PF00001	4	7tm receptor (rhodopsin family)
PF00055	4	Laminin N-terminal (Domain VI)
PF00059	4	Lectin C-type domain
PF00083	4	Sugar (and other) transporter
PF00135	4	Carboxylesterase

PfamA	#genes	description
PF00067	17	Cytochrome P450
PF00089	17	Trypsin
PF00001	14	7tm receptor (rhodopsin family)
PF00096	14	Zinc finger, C2H2 type
PF00650	14	CRAL/TRIO domain
PF07690	13	Major Facilitator Superfamily
PF00069	12	Protein kinase domain
PF00400	12	WD domain, G-beta repeat
PF00106	11	short chain dehydrogenase
PF00135	10	Carboxylesterase
PF00379	10	Insect cuticle protein
PF01061	9	ABC-2 type transporter
PF06585	9	Haemolymph juvenile horm bind prot
PF07714	9	Protein tyrosine kinase
PF00046	8	Homeobox domain
PF00201	8	UDP-glucoronosyl & -glucosyl transf
PF00520	8	Ion transport protein
PF00907	8	T-box
PF07993	8	Male sterility protein
PF00188	7	Cysteine-rich secretory protein family

Figure 4: Female antenna vs. palp enhanced gene sets. Proportional Venn diagram showing the numbers of genes that are significantly enhanced in male antenna and maxillary palps. Overlap represents the subset of genes that are significantly enhanced in both tissues. Boxes contain ranked lists of the most prevalent PfamA families in each data set.

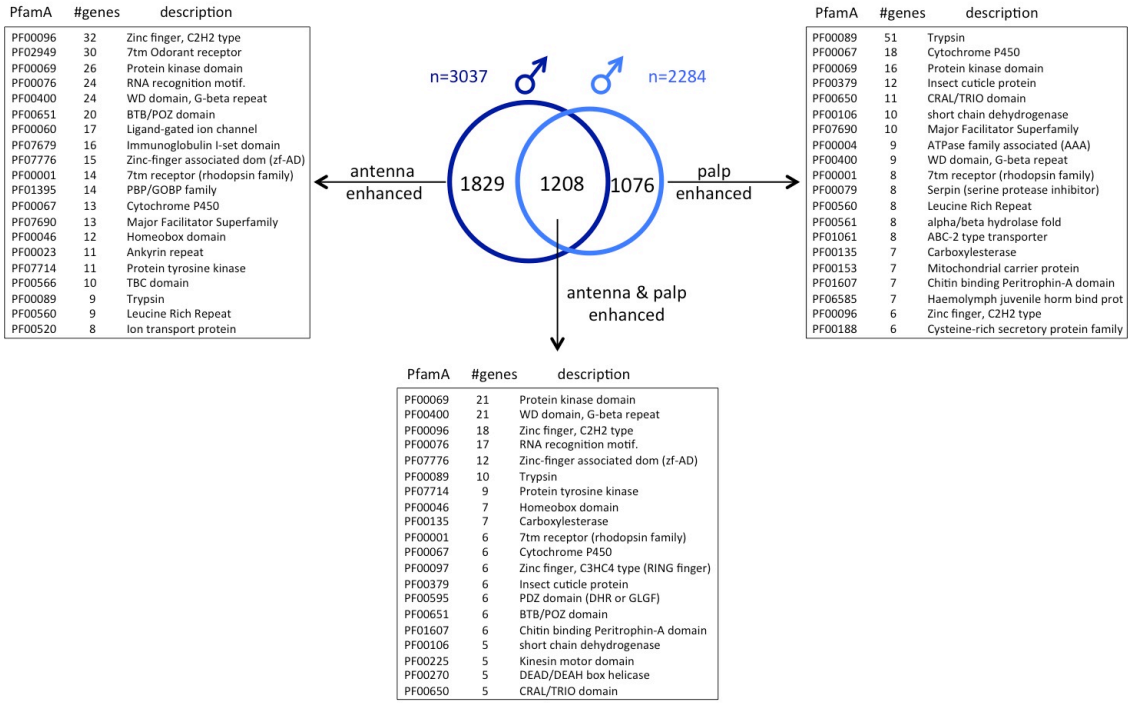


Figure 5: Male antenna vs. palp enhanced gene sets. Proportional Venn diagram showing the numbers of genes that are significantly enhanced in male antenna and maxillary palps. Overlap represents the subset of genes that are significantly enhanced in both tissues. Boxes contain ranked lists of the most prevalent PfamA families in each data set.

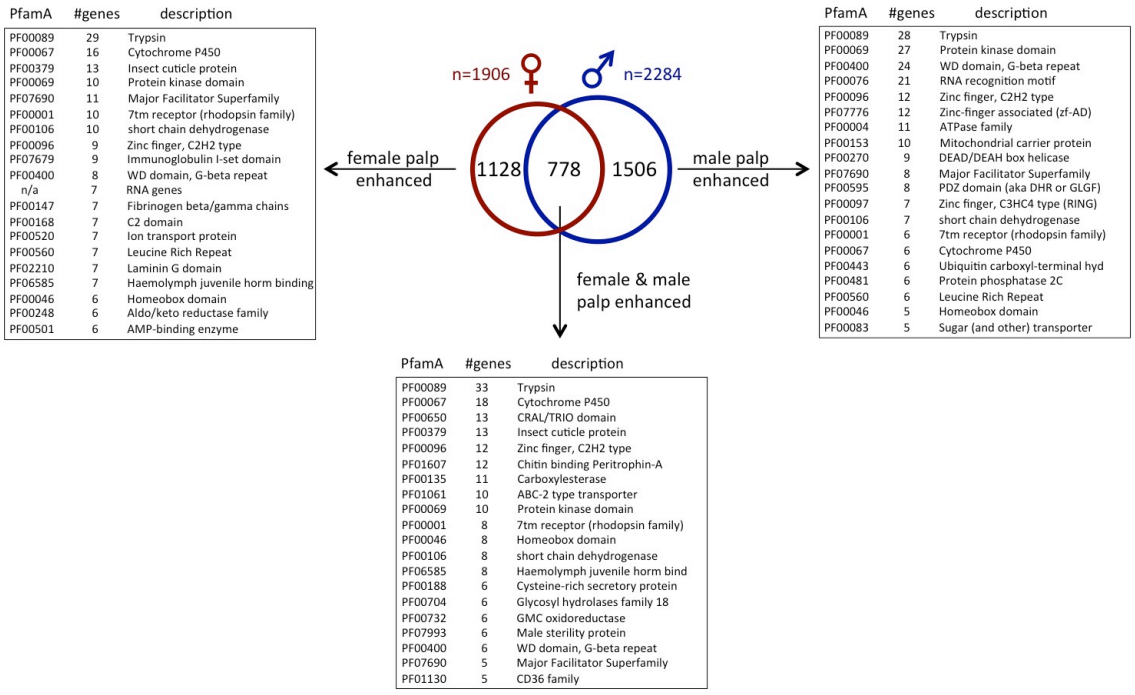


Figure 6. Female vs. male antenna enhanced gene sets. Proportional Venn diagram showing the numbers of genes that are significantly enhanced in female and male antenna. Overlap represents the subset of genes that are significantly enhanced in both sexes. Boxes contain ranked lists of the most prevalent PfamA families in each data set.

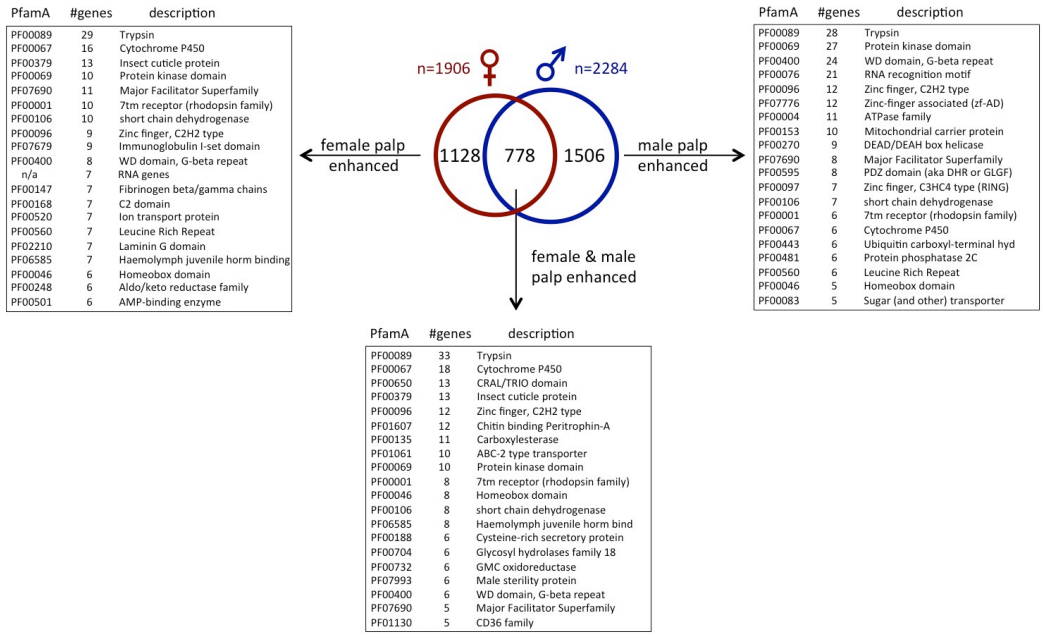


Figure 7. Female vs. male palp enhanced gene sets. Proportional Venn diagram showing the numbers of genes that are significantly enhanced in female and male maxillary palps. Overlap represents the subset of genes that are significantly enhanced in both sexes. Boxes contain ranked lists of the most prevalent PfamA families in each data set.

gambiae Ors (*AgOrs;* Hill et al., 2002; PfamA family PF02949) were the most prevelant class in female antennae (Figure 4, left table) and second-most in the male antennae (Figure 5, left table). Other chemosensory gene families, such as ligand-gated ion channels, which include the recently identified ionotropic receptors (*AgIrs;* Liu et al., 2010; PF00060), and odorant binding proteins (*AgObps*; Xu et al., 2004; PF01395) were highly represented in the antennae (Figures 4 and 5). It is clear from these antennae-to-palp analyses that both extensive overlap and significant distinctions in gene expression profiles exist. The consistent identification of the same Pfam familes in all enhanced gene sets implicates functional groups that can be studied in greater detail to elucidate their potential roles in mosquito chemosensation.

To attempt to distinguish similarities and differences in gene expression patterns between sexes, we compared the 2,277 female, and the 3,037 male antennal-enhanced genes and identified a common set of 1381 genes (Figures 3 and 6). Once again, this set included *AgOrs*, *AgIrs*, and *AgObps* (Figure 6, bottom table). Despite many commonalities in gene expression, there were also 896 female antennae-specific enhanced genes and, surprisingly, nearly 1700 male antennae-specific enhanced genes (Figures 3 and 6).

Given the obvious sexual dimorphism of *An. gambiae* antennae (Figure 7), the comparison of female to male antennae is not straightforward. Chemosensory sensilla, and *AgOr*-containing neurons in

particular, are found over the full length of the female antenna, whereas male antennae house ~3-fold fewer chemosensilla that are restricted the two most distal segments (Sutcliff 1993; McIver 1982; Ismail 1964; Schymura et al., 2010). Furthermore, while female antennae are predominantly chemosensory, male antennae are also highly specialized for hearing (Pennetier et al., 2010; Gibson et al., 2010). Accordingly, the *An. gambiae* orthologs of the *D. melanogaster trpV* channels *Nanchung* and *inactive*, which are required for hearing in the fruitfly, were enhanced in antennae of both *An. gambiae* sexes (AGAPs 012241 and 000413, respectively), but their absolute abundances were much higher in male antennae (RPKMs of 183.92 and 104.49 in males and 20.54 and 7.66 respectively, in females). This elevated abundance of auditory-associated genes in the male antenna is consistent with male *An. gambiae* mating behavior where an acute sense of hearing facilitates the recognition of female wing beats (Charlwood and Jones 1979; Pennetier et al., 2010; Gibson et al., 2010). Given that wild female mosquitoes are likely to mate just once, while males swarm daily in search of a mate (Goma 1963; Charlwood and Jones 1979; Howell and Knols 2009), the specialization shift away from olfaction and toward audition in the principle male sensory organ is reasonsable presumably as a mechanism to increase male mating success.

In the maxillary palps, as in the antennae, considerable overlap was found in gene expression profile between the sexes. In the palp, 778

genes were common between the 1,906 female palp-enhanced gene set and the 2,284 male palp-enhanced gene set (Figure 3 and Figure 8). Interestingly, the fraction of enhanced gene overlap was much lower in the palps than in the antennae (Figures 6 and 8); only 41% of the total female palp-enhanced set was shared with males, compared to 61% of the total female antennal-enhanced set that was shared with males. In light of the antennal sexual dimorphism the even greater divergence in overlapping gene sets between female and male palp may indicate the presence of cryptic sex-specific specializations.

These comparisons also revealed multiple classes of genes outside the expected chemosensory gene families that displayed enhanced tissue abundance. A detailed examination of the abundance patterns of a subset of other gene families is provided in Table 2, many of which are represented in Figures 3 and 4. Nearly half of the members of the large superfamily of 7-transmembrane (7tm) receptors (114 of the 241 recognized by PfamA), exclusive of the *AgOrs*, were enhanced in at least one of the chemosensory tissues examined (Table 2). This may indicate unrecognized roles in sensory reception or regulation of chemoreceptor neuron or accessory cell function. Importantly, efferent projections from serotonergic, or tachykinin neuroendocrine cells have been identified in mosquito chemosensory appendages (Siju et al., 2008; Meola et al., 2000, 2002). Thus the expression of serotonin (AGAPs 002232, 002679, 004222, 004223, 007136, and 011481), and tachykinin (AGAPs 001592

				Enhanced >2x ----------			
gene class	**PfamA**	**PfamA description**	***#An.gambiae***	**FA**	**MA**	**FP**	**MP**
7tm Receptor	PF00001	7tm receptor (rhodopsin family)	84	28	20	18	14
7tm Receptor	PF02949	7tm Odorant receptor (Or)	78	56	31	3	3
7tm Receptor	PF08395	7tm Chemosensory receptor (Gr)	52	1	4	3	4
7tm Receptor	PF00002	7tm receptor (Secretin family)	11	2	1	0	2
7tm Receptor	PF00003	7tm sweet-taste receptor of 3 GCPR	7	4	5	2	1
lipophilic carrier	PF01395	PBP/GOBP family	62	18	17	6	4
lipophilic carrier	PF00650	CRAL/TRIO domain	43	17	9	17	16
lipophilic carrier	PF06585	Haemolymph juvenile hormone binding	24	10	5	15	9
lipophilic carrier	PF00188	Cysteine-rich secretory protein family	20	7	2	9	7
lipophilic carrier	PF03392	Insect pheromone-bind. family, A10/OS-D	7	2	2	4	1
CD36/SNMP	PF01130	CD36 family	14	5	1	7	5
channel/transporter	PF07690	Major Facilitator Superfamily	65	21	16	16	13
channel/transporter	PF00083	Sugar (and other) transporter	49	7	4	7	8
channel/transporter	PF00060	Ligand-gated ion channel	29	22	20	5	3
channel/transporter	PF00520	Ion transport protein	27	15	10	9	3
channel/transporter	PF02931	Neurotrans.-gated ion-channel ligand bind.	24	10	6	4	0
channel/transporter	PF00858	Amiloride-sensitive sodium channel	23	5	2	1	1
channel/transporter	PF01061	ABC-2 type transporter	19	10	4	12	11
channel/transporter	PF00005	ABC transporter	18	4	3	5	2
channel/transporter	PF00664	ABC transporter transmemb.	15	4	2	2	4
channel/transporter	PF07885	Ion channel	9	3	3	1	1
biotransformation	PF00067	Cytochrome P450	113	30	19	34	24
biotransformation	PF00135	Carboxylesterase	50	15	13	14	14
biotransformation	PF00043	Glutathione S-transferase, C-term.	18	6	1	4	1
biotransformation	PF02798	Glutathione S-transferase, N-term.	17	5	3	4	3
transcription factor	PF00096	Zinc finger, C2H2 type	114	21	50	21	24
transcription factor	PF00046	Homeobox domain	76	17	19	14	13
transcription factor	PF00651	BTB/POZ domain	54	17	26	5	7
transcription factor	PF00010	Helix-loop-helix DNA-binding	41	6	6	5	6
transcription factor	PF00250	Fork head domain	19	6	8	3	4
transcription factor	PF07716	Basic region leucine zipper	14	3	4	1	3
transcription factor	PF00292	Paired box domain	10	3	5	3	3
transcription factor	PF00907	T-box	11	8	6	8	5
transcription factor	PF00170	bZIP transcription factor	8	3	3	2	2
transcription factor	PF00157	Pou domain - N-terminal to homeobox	4	2	3	3	1

Table 2. Enhanced gene classes in *An. gambiae* chemosensory tissues. Cells in each row contain information corresponding to the gene class listed. PfamA: PfamA family number. PfamA description: PfamA family description. # in *An. gambiae*: number of genes identified in PfamA searches of *An. gambiae* transcriptome. enhanced >2x: number of genes in each PfamA family that were enhanced relative to bodies in the specified tissues, relative to bodies. FA – female antenna, MA – male antenna, FP – female palp, MP – male palp

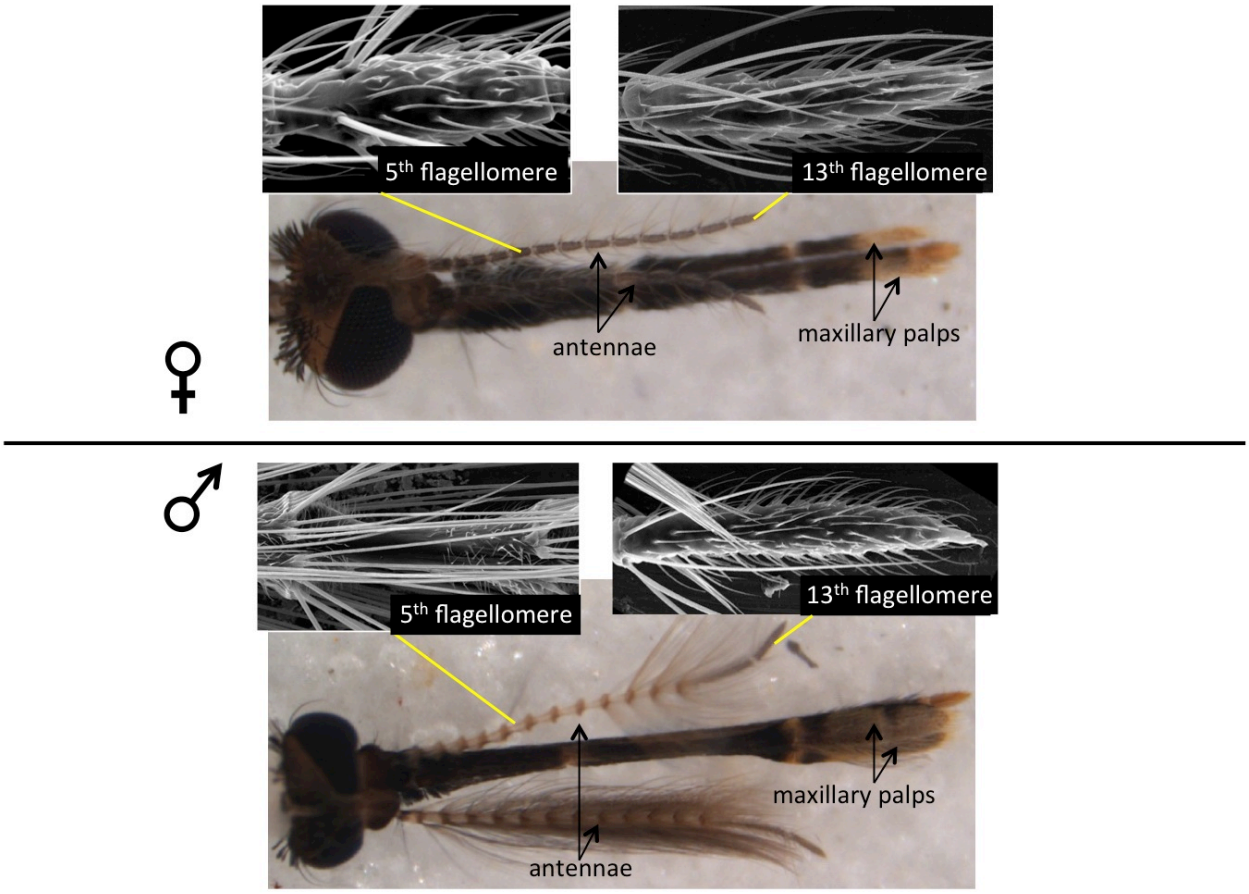


Figure 8. Sexual Dimorphism in *An. gambiae* chemosensory tissues. Brightfield images of *An. gambiae* female and male heads. Antennae and maxillary palps are indicated. Scanning electron micrographs show details of the fifth and thirteenth flagellomeres (segments) of female and male antennae, respectively.

and 012824) receptor homologs in *An. gambiae* antennae and maxillary palps is consistent with a neuromodulatory role for these compounds.

Other gene families with multiple members that displayed chemosensory enhancement include the CD36 family, some members of which function in insect olfaction (Rogers et al., 1999; Benton et al., 2009); ion channels and transporters, which include the recently identified chemosensory ionotropic receptors (Liu et al., 2010; Croset et al., 2010; Abuin et al., 2011). In addition biotransformation enzymes, such as carboxyesterases and cytochrome P450s that are potential odor degrading/biotransformation enzymes (Durand et al., 2010a, 2010b; Maibeche-Coisne et al., 2002); carbonic anhydrases involved in carbon dioxide detection in mammals (Chandrashekar J, et al., 2009) and transcription factors, including the *An. gambiae* homologs of *acj6* and *pdm3*, *D. melanogaster* pou-type transcription factors involved in *DmOr* gene regulation and ORN axon targeting (Bai and Carlson 2010; Bai et al 2009; Tichy et al., 2008; Lee and Salvaterra 2002; Clyne et al., 1999; Ayer and Carlson 1992,1991) were observed.

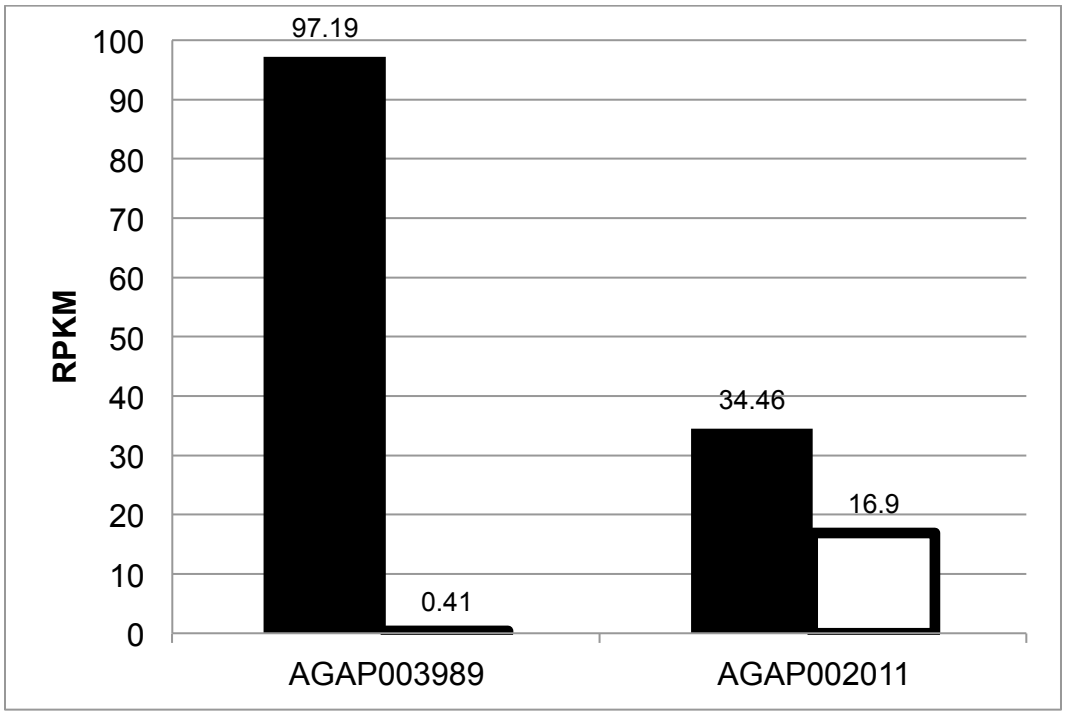
We also identified a number of small, soluble proteins with enhanced chemosensory tissue abundance in both sexes (Table 3), such as the CRAL-TRIO (PF00650) and cysteine-rich secretory (PF00188), and hemolymph juvenile hormone binding proteins (JHBP, PF06585). To our knowledge, the first two gene families have not been linked to chemosensation, but the members of the JHBP family have been

identified in screens of high abundance genes in mosquito antennae (Justice et al., 2003; Bohbot and Vogt 2005). Moreover the JHBP gene, *takeout*, links the circadian clock and feeding behavior in *D. melanogaster* (Sarvo-Blot et al., 2000) and modulates aggregation behavior in *Locusta migratoria* (Guo et al., 2011). The extremely high abundance levels of some members of these 3 gene families suggest potential chemosensory functions analogous to other soluble lipophilic carriers such as the *Obps.*

Grooved peg sensilla on mosquito antennae are sensors of many key host kairomones, including ammonia, which has been recognized as a mosquito attractant for nearly 100 years and acts as a potent synergist for eliciting host-seeking behavior (Qiu et al. 2006; Smallegange et al. 2005; Zwiebel and Takken 2004). Despite its recognized importance in host seeking and thus in human disease transmission, virtually nothing is known about the sensory signaling events that lead to ammonia perception. Importantly, grooved pegs seem to lack expression of any of the other known molecular odorant receptors that are encoded in the *An. gambiae* genome and expressed in the trichoid sensilla (Hill et al. 2002; Pitts et al. 2004). Accordingly, identifying potential kairomone receptors and especially those tuned to ammonia, that are expressed in sensilla beyond the trichoids may prove to be critical for the development of novel chemical-based control or monitoring strategies. As described in Chapter II, the *AgIrs* (Liu et al. 2010) may underlie odor sensitivities to grooved peg sensilla. The *Drosophila* homologs of the *AgIrs* are expressed in non-

trichoid sensilla and one of those receptors confers sensitivity to ammonia, although at a high threshold (Benton et al. 2009). However, there is no mosquito homolog of the *Drosophila* ammonia receptor (unpublished observation) indicating that mosquitoes are likely to utilize a novel mechanism for ammonia sensing.

Among the genes that we have identified is an ammonium transporter homolog (AGAP003989) that is highly enriched, if not exclusively expressed, in the antennae (Figure 9). In fact, the expression level of AGAP003989 in the antennae is even higher than almost any of the antennal odorant receptors (Figure 10). Ammonium transporters of the Amt and RH families are primarily known to be regulators of cellular ammonia levels in virtually all organisms (Tremblay and Hallenbeck 2009). Importantly, the expression level of a second ammonium transporter homolog in *An. gambiae* is non-tissue specific (Figure 9). More recently, ammonium transporters have been shown to carry out signaling functions in bacteria and yeast (Tremblay and Hallenbeck 2009). We speculate that AGAP003989 may participate in ammonia sensitivity in the grooved peg sensilla of *An. gambiae* and other mosquitoes. In support of the second conjecture, we have identified orthologs of AGAP003989 in the genomes of *Aedes aegypti* and *Culex quinquefasciatus* (Figure 9). AGAP003989 therefore represents a potentially novel and important finding that whole transcriptome profiling has facilitated.



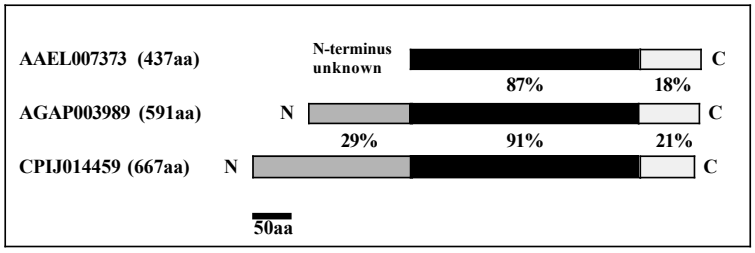


Figure 9. Top panel - Expression of ammonium transporter homologs in *An. gambiae* female antennae (black bars) and bodies (white bars). Bottom panel - Schematic representation of ammonium transporter orthologs from *Ae. aegypti*, *An. gambiae*, and *Cx. quinquefasciatus*. Peptides are represented as shaded boxes with regions of homology indicated in different shades. Percent identical residues are listed

Chemosensory Gene Families

In light of the existing literature on the molecular mechanisms underlying the processes of peripheral chemosensation in vector mosquitoes, we examined in detail the abundance patterns *AgOrs*, *AgIrs*, *AgObps* and gustatory receptors (*AgGrs*). As expected, the vast majority of *AgOrs* were highly enhanced in antennae. Of the 76 *AgOrs,* 58 showed significantly higher expression in female antennae as compared to only 36 in male antennae (Figure 10). The entire set of male-enhanced *AgOr*s was contained within the female enhanced set. None of the larval-specific *AgOrs: 37, 40, 52,* or *58,* was enhanced in adult antennae or palps, supporting previous observations (Xia et al., 2008). In the palps, only *AgOrs8* and *28* and *AgOrco* (recently renamed from *AgOr7* to reflect its capacity as an obligate co-receptor in *Or* signaling) were enhanced in female maxillary palps (Figure 10), a result consistent with our previous study on odor coding in the *An. gambiae* maxillary palps (Lu et al., 2007). The same 3 *AgOrs* were enhanced in male palps (Figure 10).

Several members of the recently described *AgIr* gene family (Liu et al., 2010; Croset et al., 2010) displayed significant enhancement in antennae of both sexes (Figure 11), further supporting their potential roles as chemosensory receptors in *An. gambiae*. A high degree of overlap was observed between the sexes, where 21 *AgIrs* were enhanced in both. Similar to the *AgOrs*, there were many fewer *AgIrs* enhanced in the palps

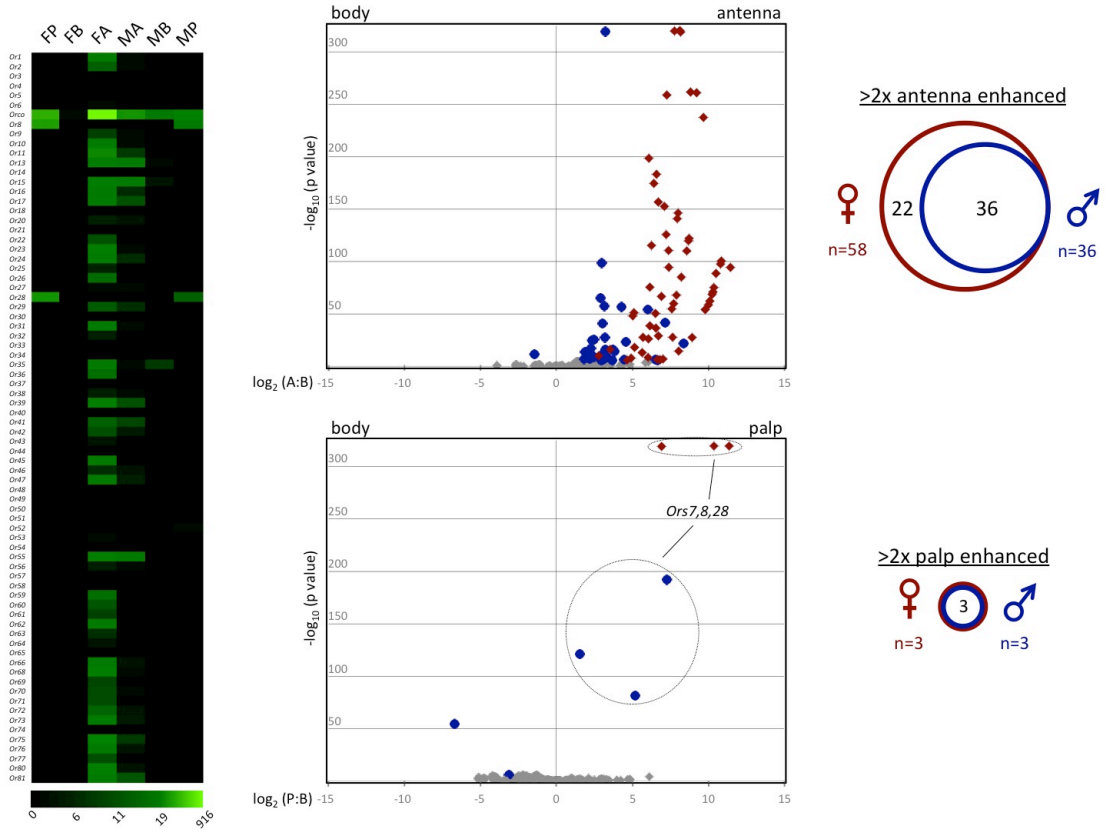


Figure 10. *AgOr* Expression Profile. Left panel is an expression profile map. Green color intensity scale (below map) indicates increasing RPKM values from left to right. (FP – female palp; FB – female body; FA – female antenna; MA – male antenna; MB – male body; MP – male palp). Middle panels – volcano plots showing the relative abundance of *AgOrs* in body versus antennae. Individual data points were plotted at the intersection of the $\log_{10}$ of Fisher's exact test (y-axis) and the $\log_2$ of the ratio of antenna (or palp) RPKM: body RPKM (x-axis) for each gene. Red diamonds or blue circles represent significantly enhanced *AgOrs* in antenna (top panel) or maxillary palps (bottom panel) of females and males, respectively. Gray points represent *AgOrs* that fell below the significance threshold of 3.91e-06 or the 2-fold differential expression cutoff. RPKM values of 0.00 were transformed to 0.10 prior to calculating RPKM ratios, such that those genes could also be represented on the plot. Right panels – Proportional Venn diagrams showing the number of *AgOrs* that are significantly enhanced in female and male antenna (top) and maxillary palp (bottom).

compared to the antennae, with 7 and 6 enhanced in female and male palps, respectively. Furthermore, the degree of overlap (3 genes) between the sexes was much less pronounced in the palp (Figure 11).

The *AgGrs* were the only class that did not overlap in the antennae between the sexes, with very few showing enhancement in either females or males (Figure 12). Only *AgGr1* was enhanced in female antennae, while *AgGrs, 33, 48, 49,* and *50* were enhanced in male antennae. Notably, one member of this large gene family, *AgGr33* was strongly enhanced in the male antenna (Figure 12), perhaps indicating a specialized function in male antennae. In contrast to the acute sexual dimorphism displayed in the antennae, both sexes showed high abundance of *AgGrs 22*, *23*, and *24*, in their maxillary palps (Figure 12). These three *AgGrs* are homologs of the *D. melanogaster* carbon dioxide receptors (Kwon et al., 2007; Cayirlioglu et al., 2008; Robertson and Kent 2009) and are expressed in capitate peg sensilla on the maxillary palps where they have been directly implicated in *An. gambiae* CO_2 sensing (Lu et al., 2007).

Enhanced chemosensory abundance of members of the large *AgObp* family was evident across all tissues and sexes (Figure 13). Sixteen classical and three C-plus *AgObp*s were significantly enhanced in the female antennae (Figure 13). Of these, 17 were also significantly enhanced in the male antennae (Figure 13) including the LUSH homolog, *AgObp4* (Kim et al., 1998). *AgObp19* was the only one to demonstrate

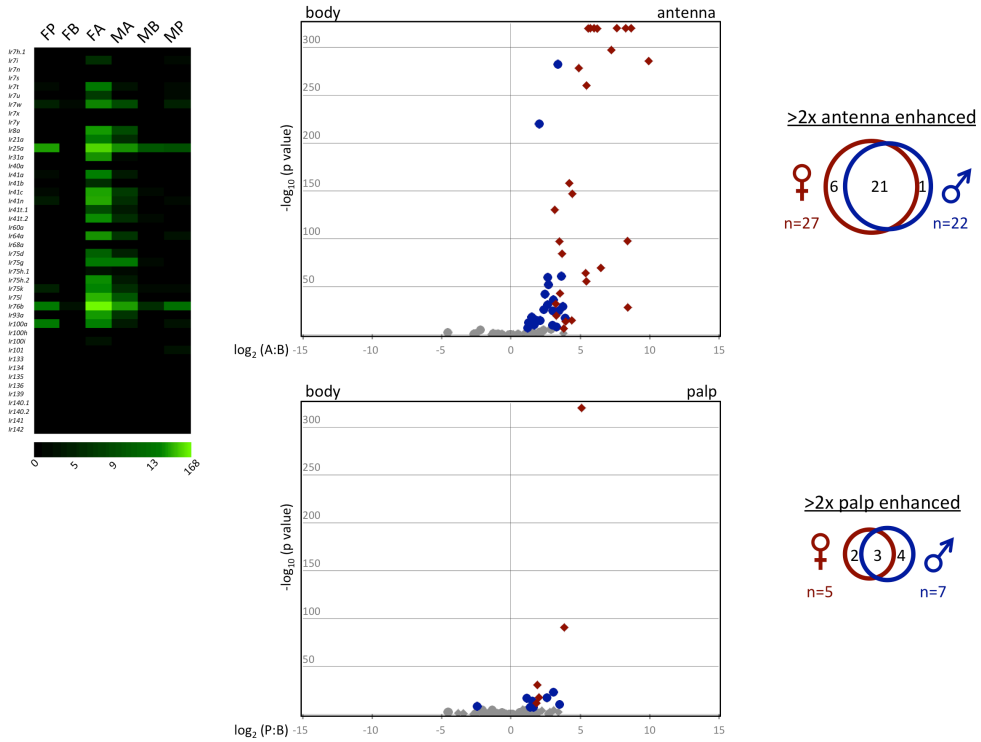


Figure 11. *Aglr* Expression Profile. Left panel is an expression profile map. Green color intensity scale (below map) indicates increasing RPKM values from left to right. Column labels same as Figure 9. Middle panels – volcano plots showing the relative abundance of *Aglrs* in body versus antennae. Individual data points were plotted at the intersection of the $\log_{10}$ of Fisher's Exact test (y-axis) with the $\log_2$ of the ratio of antenna (or palp) RPKM: body RPKM (x-axis) for each gene. Red diamonds or blue circles represent significantly enhanced *Aglrs* in antenna (top panel) or maxillary palps (bottom panel) of females and males, respectively. Gray points represent *Aglrs* that fell below the significance threshold of 3.91e-06 or the 2-fold differential expression cutoff. RPKM values of 0.00 were transformed to 0.10 prior to calculating abundance ratios, such that those genes could also be represented on the plot. Right panels – Proportional Venn diagrams showing the number of *Aglrs* that are significantly enhanced in female and male antenna (top) or palp (bottom).

significantly enhanced abundance in the female antennae and in no other tissue. In the maxillary palp, enhancement of *AgObp* transcripts also displayed substantial overlap between sexes, where the 4 male enhanced *AgObps* were all enhanced in females. Overall, *AgObp* abundance was nearly identical between male and female chemosensory tissues (Figure 13).

In contrast, atypical *AgObp*s were not enhanced in any of the tissues examined, which is consistent with previous results suggesting that expression of this subfamily is limited to pre-adult stages (Xu et al., 2005). With the exception of *AgObps 47*, *48*, *57*, which had RPKMs of >1000, abundance of the members of the Plus-C *AgObp* subfamily was very low. Of these, it is noteworthy that *AgObp48* was one of the most highly expressed genes (RPKM=32311) in any tissue, with significant abundance in both the male and female olfactory tissues. While *AgObps*, and insect *Obps* in general are among the most highly expressed gene families in chemosensory tissues their role in non-pheromone chemosensation remains largely undefined.

It has been hypothesized that *Obps* act as molecular shuttles/chaperones, which deliver to receptors and/or transiently protect specific odorants from biotransformation enzymes (Vogt, 1987 Lerner et al., 1990). If individual *Obps* bind a subset of odorants, it is reasonable to hypothesize that in tissues with high *Or* and therefore odor-coding complexity such as the antennae, the *Obp* landscape would need to be

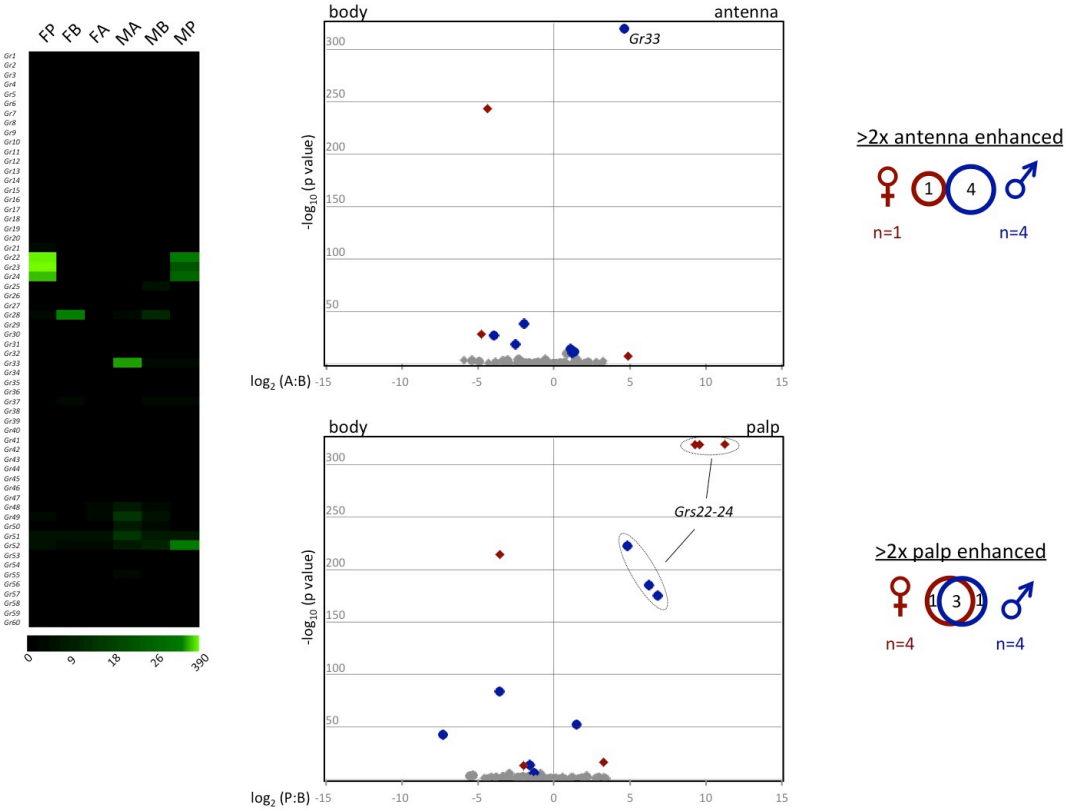


Figure 12. *AgGr* Expression Profile. Left panel is an expression profile map. Green color intensity scale (below map) indicates increasing RPKM values from left to right. Column labels same as Figure 9. Middle panels – volcano plots showing the relative abundance of *AgGrs* in body versus antennae. Individual data points were plotted at the intersection of the $\log_{10}$ of Fisher's exact test (y-axis) with the $\log_2$ of the ratio of antenna (or palp) RPKM: body RPKM (x-axis) for each gene. Red diamonds or blue circles represent significantly enhanced *AgGrs* in antenna (top panel) or maxillary palp (bottom panel) of females and males, respectively. Gray points represent *AgGrs* that fell below the significance threshold of 3.91e-06 or the 2-fold differential expression cutoff. RPKM values of 0.00 were transformed to 0.10 prior to calculating expression ratios, such that those genes could also be represented on the plot. Right panels – Venn diagrams showing the number of *AgGrs* that are significantly enhanced in female and male antenna (top) or palp (bottom).

similarly complex in order to bind the required range of odorants. The converse would also be expected for tissues with reduced odor coding complexity such as the maxillary palp.

The female antenna expresses transcripts for 58 conventional *AgOrs* whose levels are significantly enhanced over the body, while the female palp expresses only 3. Furthermore, the odorant response profiles of the palp- expressed *AgOrs*8 and *28* are also vastly different from the de-orphanized antennal *AgOrs* (Lu et al., 2007; Carrey et al., 2010; Wang et al., 2010). These differences in *AgOr* coding capacity and their expression profiles strongly suggest that the ability of the female antennae to sense odors is much greater than the maxillary palp.

In *An. gambiae* females both the antennae and maxillary palps expressed 21 *AgObp* family members with an RPKM >10, of which 19 were found in both (Figure 13). While not all of these *AgObps'* abundance levels meet our significance criteria for enhancement, these genes are nevertheless expressed in these tissues. Thus although the *AgObp* complexity is almost identical, these two appendages, display a vastly different *AgOr* complexity and odor coding capacity (odor space). This analysis confounds standing theories about *Obp* function; if all antennal *Obps* are required for signaling, then their presence in the palp, with its much more limited odor space, would appear superfluous. Given this broad expression, and a demonstrated lack of functional overlap, this analysis instead suggests that in at least some instances, *Obps* act as

low-pass filters for environmental odorants rather than as specific odorant-carrier agents. Therefore, *Obps* may act to solubilize odors in some cases, but as molecular sinks in others, adding yet another dimension to peripheral odor coding.

Diverse Roles for Chemosensory Tissues

To explore the effect of morphology on observed *AgOr* expression, we attempted to normalize sex-specific differences in transcript abundance by scaling up male *AgOrs* in proportion to the number of female chemosensilla. *AgOrs* are known to be expressed in the trichoid sensilla, the predominant sensillar type and not in grooved peg sensilla (Pitts et al., 2004). Sensilla counts indicate that female antennae house an average of 630 trichoid sensilla while male antennae house an average of 225 trichoid sensilla (Pitts and Zwiebel 2006; McIver et al., 1982; Ismail 1964). We therefore multiplied the male *AgOr* RPKMs by a factor of 630/225 or 2.8. After normalizing, *AgOr* expression profiles were qualitatively very similar in females and males (Figure 14), although the male *AgOr* RPKM values remained lower than those in females. Based on the same logic, we also normalized *AgIr* expression in male antennae (Figure 14). Because we postulated that *AgIrs* are localized in neurons housed in grooved peg sensilla (GP) as they are in *D. melanogaster* (Benton et al., 2009; Liu et al., 2010), we used a GP normalization factor of 4.2, which is the fold difference in GP numbers between female and

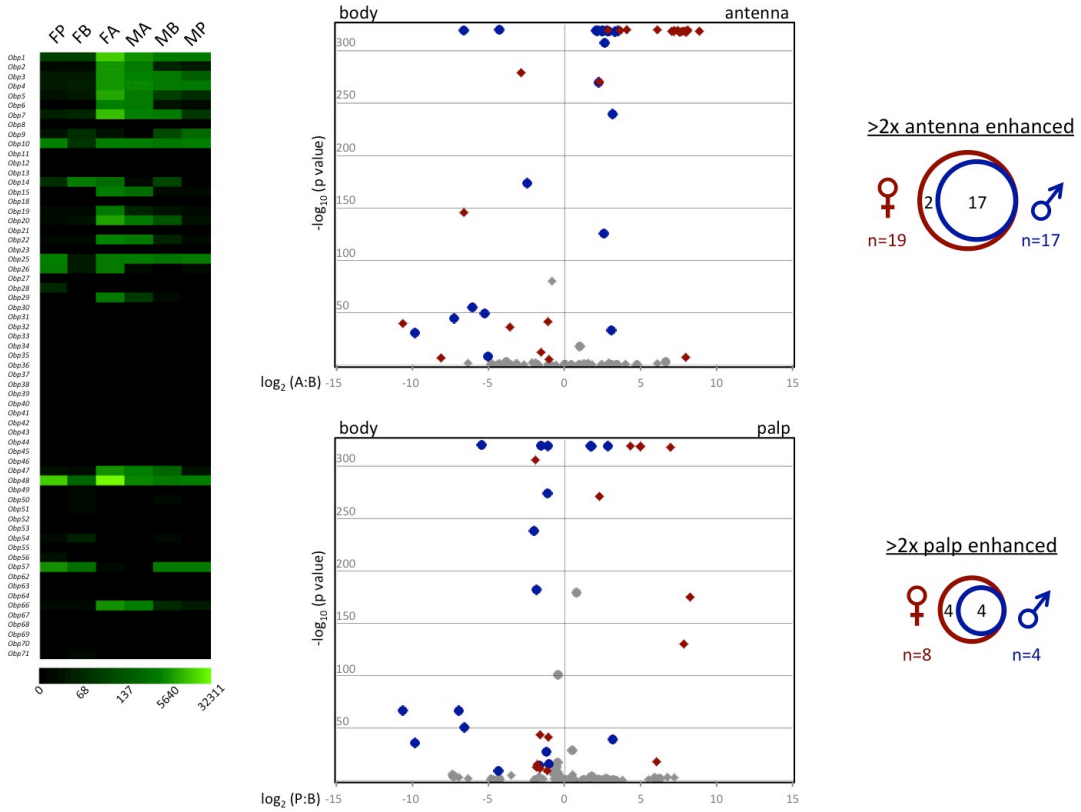


Figure 13. *AgObp* Expression Profile. Left panel is an expression profile map. Green color intensity scale (below map) indicates increasing RPKM values from left to right. Column labels same as Figure 9. Middle panels – volcano plots showing the relative abundance of *AgObps* in body versus antennae. Individual data points were plotted at the intersection of the $\log_{10}$ of Fisher's exact test (y-axis) with the $\log_2$ of the ratio of antenna (or palp) RPKM: body RPKM (x-axis) for each gene. Red diamonds or blue circles represent significantly enhanced *AgObps* in antenna (top panel) or maxillary palps (bottom panel) of females and males, respectively. Gray points represent *AgObps* that fell below the significance threshold of 3.91e-06 or the 2-fold differential expression cutoff. RPKM values of 0.00 were transformed to 0.10 prior to calculating expression ratios, such that those genes could also be represented on the plot. Right panels – Venn diagrams showing the number of *AgObps* that are significantly enhanced in female and male antenna (top) or palp (bottom).

male *An. gambiae* antennae (McIver 1982). As with *AgOrs*, the *AgIr* gene expression patterns were qualitatively similar in both sexes after normalization (Figure 14). These results suggest that male antennae express similar *AgOr* and *AgIr* chemoreceptor repertoires as the female antennae, although, importantly, at reduced absolute levels.

The *AgOr* and *AgGr* abundance profiles in the maxillary palps support a similar conclusion. Although *AgOrs 7, 8,* and *28* and *AgGrs22-24* were enhanced in both sexes, their abundance levels were lower in males than in females (Figures 10 and 12). As is the case for *An. gambiae* antennae, the maxillary palps are sexually dimorphic and in males they house about 4-fold fewer chemosensilla (McIver 1982; Lu et al., 2007). This could account for the apparent lower chemosensory gene transcript abundances in males. Normalizing male palp *AgOrs* and *AgGrs* by this factor brings their absolute RPKM values closer to those of females, but does not affect the qualitative observation that the identical chemoreceptors are enhanced there (data not shown). The same can be said for *AgObps* in the antennae and maxillary palps (Figures 11 and 13), where this gene family is generally more enhanced in females than in males. Assuming that the transcript abundance profiles seen here are meaningful at the functional level, both sexes would potentially be receptive to a qualitatively similar odor space, with females perhaps having a lower threshold response to odors and thus greater chemoreceptive power. In either case, the aforementioned differences in

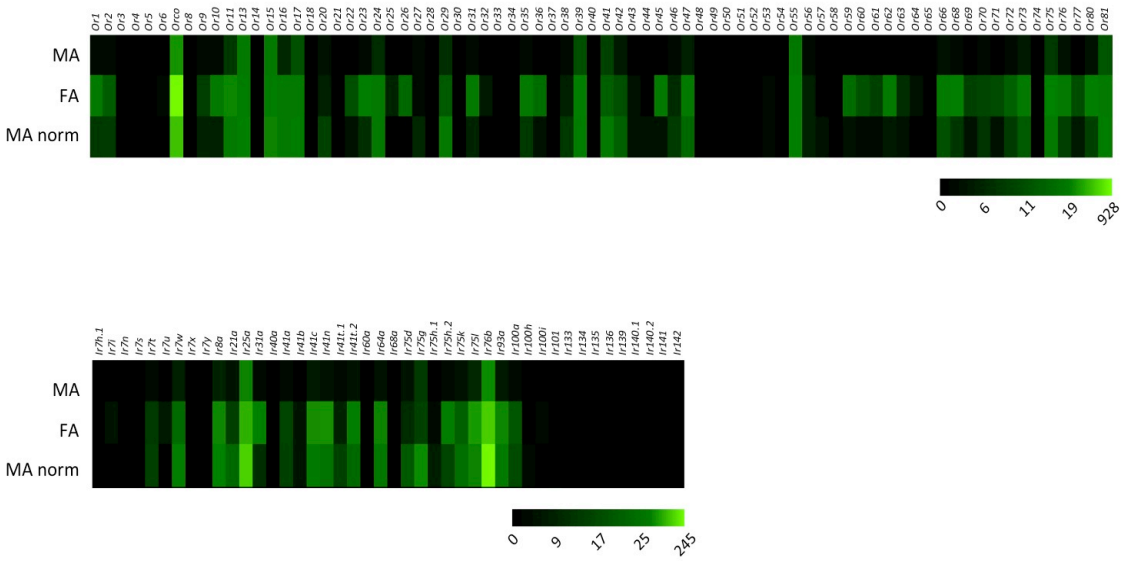


Figure 14. Sensilla-normalized *AgOr* and *AgIr* Expression Profiles. Expression profile maps using a green color scale to represent RPKM values for *AgOr* (top panel) and *AgIr* (bottom panel) families. MA – male antenna RPKMs. FA – female antennae RPKMs. MA norm – male antenna normalized RPKMs. Male antennae *AgOrs* were scaled up by a factor of 2.8. Male antennae *AgIrs* were scaled up by a factor of 4.2. Scales shown below expression maps. Color scales shown below maps indicate increasing RPKM values from right to left.

gene abundance profiles could also be functionally relevant and serve as the basis for distinguishing qualitatively and quantitatively female and male chemosensory abilities. These competing hypotheses are directly testable using a combination of electrophysiological recording and behavioral response assays. Moreover, the requirement in chemoreception for any of the differentially expressed genes could potentially be explored by gene silencing.

AgOr Expression in Male Bodies

We observed enhanced expression of a small number of *AgOrs* in male bodies as compared to antennae or palps (Figure 15). When we compared the *AgOr* expression patters in male and female bodies directly, a surprising result appeared. 5 *AgOrs* (*7*, *13*, *15*, *24*, and *35*) were enhanced in male bodies. This raises the exciting possibility that males have a cryptic chemosensory capacity that is lacking in females. Interestingly we were able to amplify both *AgOr7* and *AgOr35* in RT-PCRs using cDNA isolated from male reproductive tissues (Figure 16). This is a potentially important outcome of our analyses and indicates another level of discovery that whole transcriptome surveys can provide. Further study is required to elucidate the nature of this difference, but we speculate that *AgOrs* might be expressed in sperm and mediate chemotaxis toward spermatheca or oocytes. Such mechanisms are thought to be important in

mammals, where sperm have been shown to express odorant receptors and chemotax toward their cognate ligands (Spehr et al. 2004; 2006).

Conclusion

We are interested in understanding the molecular components of the chemosensory pathways that distinguish blood-feeding, female mosquitoes that carry out disease-transmission and males that do neither. Considerable effort has been devoted to catalog the semiochemicals released by potential blood-meal hosts that act as attractive signals for female mosquitoes (Takken and Knols 1999; Zwiebel and Takken 2004) as well understand the odorant response profiles of *AgOrs* (Lu et al., Carey et al., 2010; Wang et al., 2010). Differential gene expression between the sexes may serve as a potential mechanism for modulating peripheral sensitivity. Thus we have carried out a comprehensive comparative analysis of the chemosensory transcriptomes of adult male and female *An. gambiae*. In addition to identifying genes that may function in sexually differential responses, this analysis has revealed many genes that are enhanced in the antennae and maxillary palps of both sexes and are therefore likely to play essential roles in maintaining neuronal and or chemosensory functionality.

RNA sequencing has provided unparalleled resolution for the examination of global gene expression profiles in chemosensory tissues and bodies of an organism of great medical importance. By their very

nature, this data are not only broad, covering nearly the entire transcriptome of the organism, but deep, allowing one to observe not only gene expression patterns, but address questions regarding gene structure, alternative splicing, and polymorphisms to name just a few possibilities. This study has begun to explore the potential of this data set and establishes an important precedent in the use of RNA-seq for the study of chemosensation in a disease vector.

References

Abuin L, Bargeton B, Ulbrich MH, Isacoff EY, Kellenberger S, and Benton S (2011) Functional architecture of olfactory ionotropic glutamate receptors. *Neuron* 69: 44-60.

Aguilar R, Jedlicka AE, Mintz M, Mahairaki V, Scott AL, and Dimopoulos G (2005) Global gene expression analysis of *Anopheles gambiae* responses to microbial challenge. *Insect Biochem Mol Biol* 35: 709-719.

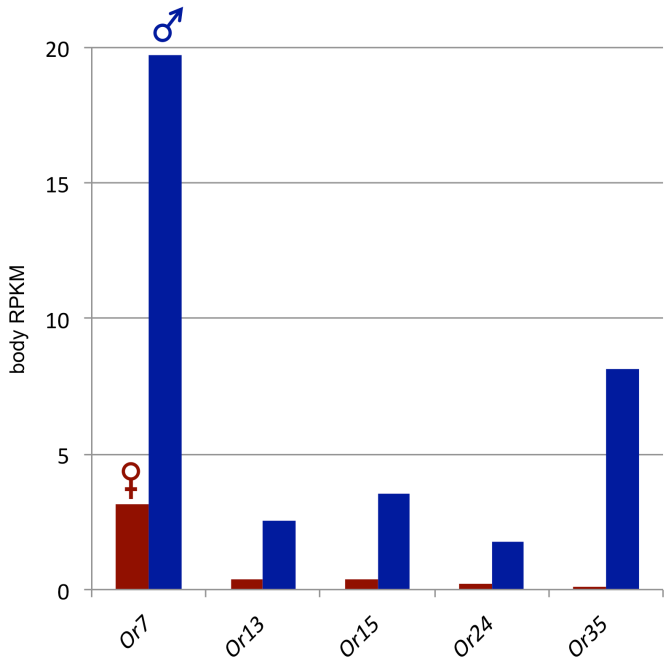
Aguilar R, Simard F, Kamdem C, Shields T, Glass GE, Garver LS, and Dimopoulos G (2010) Genome-wide analysis of transcriptomic divergence between laboratory colony and field *Anopheles gambiae* mosquitoes of the M and S molecular forms. *Insect Mol Biol* 19: 695-705.

Ayer RK, Jr. and Carlson J (1991) *acj6*: a gene affecting olfactory physiology and behavior in *Drosophila*. *Proc Natl Acad Sci U S A* 88: 5467-5471.

Ayer RK, Jr. and Carlson J (1992) Olfactory physiology in the *Drosophila* antenna and maxillary palp: *acj6* distinguishes two classes of odorant pathways. *J Neurobiol* 23: 965-982.

Bai L and Carlson JR (2010) Distinct functions of *acj6* splice forms in odor receptor gene choice. *J Neurosci* 30: 5028-5036.

Figure 15. *AgOr* expression in *An. gambiae* bodies. RPKM values for



AgOrs 7, 13, 15, 24, and *35* in female (red bars) and male (blue bars) bodies.

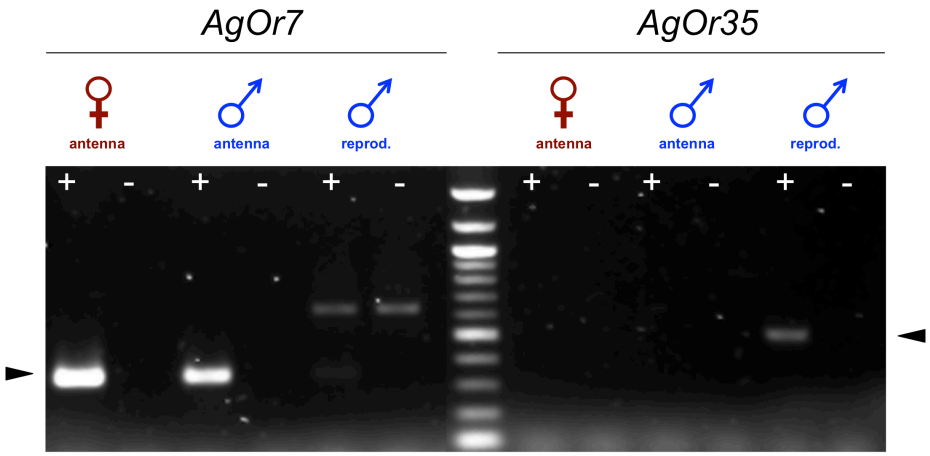


Figure 16. *AgOr* expression in *An. gambiae* male reproductive organs. Agarose gel showing RT-PCR amplicons for *AgOrs 7* and *35* in female and male tissues. Arrows indicate expected sizes of cDNAs for *AgOr7* (346bp) and *AgOr35* (526bp). Plus (+): amplification using cDNAs synthesized with reverse transcriptase. Minus (-): amplification using negative control cDNAs synthesized without reverse transcriptase.

Bai L, Goldman AL, and Carlson JR (2009) Positive and negative regulation of odor receptor gene choice in *Drosophila* by *acj6*. *J Neurosci* 29: 12940-12947.

Balwierz PJ, Carninci P, Daub CO, Kawai J, Hayashizaki Y, Van Belle W, Beisel C, and van Nimwegen E (2009) Methods for analyzing deep sequencing expression data: constructing the human and mouse promoterome with deepCAGE data. *Genome Biol* 10: R79.

Baton LA, Robertson A, Warr E, Strand MR, and Dimopoulos G (2009) Genome-wide transcriptomic profiling of *Anopheles gambiae* hemocytes reveals pathogen-specific signatures upon bacterial challenge and *Plasmodium berghei* infection. *BMC Genomics* 10: 257.

Benton R, Vannice KS, Gomez-Diaz C, and Vosshall LB (2009) Variant ionotropic glutamate receptors as chemosensory receptors in *Drosophila*. *Cell* 136: 149-162.

Bohbot J and Vogt RG (2005) Antennal expressed genes of the yellow fever mosquito (*Aedes aegypti* L.); characterization of *odorant-binding protein 10* and *takeout*. *Insect Biochem Mol Biol* 35: 961-979.

Bohbot JD, Jones PL, Wang G, Pitts RJ, Pask GM, and Zwiebel LJ (2011) Conservation of indole responsive odorant receptors in mosquitoes reveals an ancient olfactory trait. *Chem Senses* 36: 149-160.

Bonizzoni M, Dunn WA, Campbell CL, Olson KE, Dimon MT, Marinotti O, and James AA (2011) RNA-seq analyses of blood-induced changes in gene expression in the mosquito vector species, *Aedes aegypti*. *BMC Genomics* 12: 82.

Carey AF, Wang G, Su CY, Zwiebel LJ, and Carlson JR (2010) Odorant reception in the malaria mosquito *Anopheles gambiae*. *Nature* 464: 66-71.

Cayirlioglu P, Kadow IG, Zhan X, Okamura K, Suh GS, Gunning D, Lai EC, and Zipursky SL (2008) Hybrid neurons in a microRNA mutant are putative evolutionary intermediates in insect CO2 sensory systems. *Science* 319: 1256-1260.

Chandrashekar J, Yarmolinsky D, von Buchholtz L, Oka Y, Sly W, Ryba NJ, and Zuker CS (2009) The taste of carbonation. *Science* 326: 443-445.

Charlwood JD and Jones MDR (1979) Mating behaviour in the mosquito, *Anopheles gambiae* s.l. Close range and contact behaviour. *Physiological Entomology* 4: 111-120.

Clements AN (1992) **The Biology of Mosquitoes**. London, Glasgow, New York, Tokyo, Melbourne, Madras: Chapman & Hall. 509 p.

Clyne PJ, Certel SJ, de Bruyne M, Zaslavsky L, Johnson WA, and Carlson JR (1999) The odor specificities of a subset of olfactory receptor neurons are governed by *Acj6*, a POU-domain transcription factor. *Neuron* 22: 339-347.

Cook PE and Sinkins SP (2010) Transcriptional profiling of *Anopheles gambiae* mosquitoes for adult age estimation. *Insect Mol Biol* 19: 745-751.

Costantini C, Sagnon N, della Torre A, and Coluzzi M (1999) Mosquito Behavioral Aspects of Vector-Human Interactions in the *Anopheles gambiae* complex. *Parassitologia* 41: 209-217.

Crawford JE, Guelbeogo WM, Sanou A, Traore A, Vernick KD, Sagnon N, and Lazzaro BP (2010) De novo transcriptome sequencing in *Anopheles funestus* using Illumina RNA-seq technology. *PLoS One* 5: e14202.

Croset V, Rytz R, Cummins SF, Budd A, Brawand D, Kaessmann H, Gibson TJ, and Benton R (2010) Ancient protostome origin of chemosensory ionotropic glutamate receptors and the evolution of insect taste and olfaction. *PLoS Genet* 6: e1001064.

Das S, Radtke A, Choi YJ, Mendes AM, Valenzuela JG, and Dimopoulos G (2010) Transcriptomic and functional analysis of the *Anopheles gambiae* salivary gland in relation to blood feeding. *BMC Genomics* 11: 566.

de Bruyne M and Baker TC (2008) Odor detection in insects: volatile codes. *J Chem Ecol* 34: 882-897.

della Torre A, Favia G, Mariotti G, Coluzzi M, and Mathiopoulos KD (1996) Physical map of the malaria vector *Anopheles gambiae*. *Genetics* 143: 1307-1311.

Durand N, Carot-Sans G, Chertemps T, Bozzolan F, Party V, Renou M, Debernard S, Rosell G, and Maibeche-Coisne M (2010) Characterization of an antennal carboxylesterase from the pest moth *Spodoptera littoralis* degrading a host plant odorant. *PLoS One* 5: e15026.

Durand N, Carot-Sans G, Chertemps T, Montagne N, Jacquin-Joly E, Debernard S, and Maibeche-Coisne M (2010) A diversity of putative carboxylesterases are expressed in the antennae of the noctuid moth *Spodoptera littoralis*. *Insect Mol Biol* 19: 87-97.

Finn RD, Mistry J, Tate J, Coggill P, Heger A, Pollington JE, Gavin OL, Gunasekaran P, Ceric G, Forslund K, Holm L, Sonnhammer EL, Eddy SR, and Bateman A (2010) The Pfam protein families database. *Nucleic Acids Res* 38: D211-222.

Fox AN, Pitts RJ, Robertson HM, Carlson JR, and Zwiebel LJ (2001) Candidate odorant receptors from the malaria vector mosquito *Anopheles gambiae* and evidence of down-regulation in response to blood feeding. *Proc Natl Acad Sci U S A* 98: 14693-14697.

Gibbons JG, Janson EM, Hittinger CT, Johnston M, Abbot P, and Rokas A (2009) Benchmarking next-generation transcriptome sequencing for functional and evolutionary genomics. *Mol Biol Evol* 26: 2731-2744.

Gibson G, Warren B, and Russell IJ (2010) Humming in tune: sex and species recognition by mosquitoes on the wing. *J Assoc Res Otolaryngol* 11: 527-540.

Gillot C (2005) **Entomology 3rd Edition**: Springer.

Gong Z, Son W, Chung YD, Kim J, Shin DW, McClung CA, Lee Y, Lee HW, Chang DJ, Kaang BK, Cho H, Oh U, Hirsh J, Kernan MJ, and Kim C (2004) Two interdependent TRPV channel subunits, *inactive* and *Nanchung*, mediate hearing in *Drosophila*. *J Neurosci* 24: 9059-9066.

Guo W, Wang X, Ma Z, Xue L, Han J, Yu D, and Kang L (2011) CSP and Takeout Genes Modulate the Switch between Attraction and Repulsion during Behavioral Phase Change in the Migratory Locust. *PLoS Genet* 7: e1001291.

Hallem E, Ho MG, and Carlson JR (2004) The Molecular Basis of Odor Coding in the *Drosophila* Antenna. *Cell* 117: 965-979.

Hallem EA and Carlson JR (2006) Coding of odors by a receptor repertoire. *Cell* 125: 143-160.

Hekmat-Scafe DS, Scafe CR, McKinney AJ, and Tanouye MA (2002) Genome-wide analysis of the odorant-binding protein gene family in *Drosophila melanogaster*. *Genome Res* 12: 1357-1369.

Hill CA, Fox AN, Pitts RJ, Kent LB, Tan PL, Chrystal MA, Cravchik A, Collins FH, Robertson HM, and Zwiebel LJ (2002) G protein-coupled receptors in *Anopheles gambiae*. *Science* 298: 176-178.

Hittinger CT, Johnston M, Tossberg JT, and Rokas A (2010) Leveraging skewed transcript abundance by RNA-Seq to increase the genomic depth of the tree of life. *Proc Natl Acad Sci U S A* 107: 1476-1481.

Howell PI and Knols BG (2009) Male mating biology. *Malar J* 8 Suppl 2: S8.

Ismail IA (1964) Comparative Study of Sense Organs in the Antennae of Culicine and Anopheline Female Mosquitoes. *Acta Trop* 21: 155-168.

Justice RW, Dimitratos S, Walter MF, and Biessmann H (2003) Sexual Dimorphic Expression of Putative Antennal Carrier Protein Genes in the Malaria Vector *Anopheles gambiae*. *Insect Mol Biol* 12: 581-594.

Kaissling KE (2009) Olfactory perireceptor and receptor events in moths: a kinetic model revised. *J Comp Physiol A: Neuroethol Sens Neural Behav Physiol* 195: 895-922.

Kim J, Chung YD, Park DY, Choi S, Shin DW, Soh H, Lee HW, Son W, Yim J, Park CS, Kernan MJ, and Kim C (2003) A TRPV family ion channel required for hearing in *Drosophila*. *Nature* 424: 81-84.

Kim MS, Repp A, and Smith DP (1998) LUSH odorant-binding protein mediates chemosensory responses to alcohols in *Drosophila* melanogaster. *Genetics* 150: 711-721.

Kruse SW, Zhao R, Smith DP, and Jones DN (2003) Structure of a specific alcohol-binding site defined by the odorant binding protein LUSH from *Drosophila melanogaster*. *Nat Struct Biol* 10: 694-700.

Kwon HW, Lu T, Rutzler M, and Zwiebel LJ (2006) Olfactory responses in a gustatory organ of the malaria vector mosquito *Anopheles gambiae*. *Proc Natl Acad Sci U S A* 103: 13526-13531.

Kwon JY, Dahanukar A, Weiss LA, and Carlson JR (2007) The molecular basis of CO2 reception in *Drosophila*. *Proc Natl Acad Sci U S A* 104: 3574-3578.

Lee MH and Salvaterra PM (2002) *Abnormal chemosensory jump 6* is a positive transcriptional regulator of the cholinergic gene locus in *Drosophila* olfactory neurons. *J Neurosci* 22: 5291-5299.

Li J, Riehle MM, Zhang Y, Xu J, Oduol F, Gomez SM, Eiglmeier K, Ueberheide BM, Shabanowitz J, Hunt DF, Ribeiro JM, and Vernick KD (2006) *Anopheles gambiae* genome reannotation through synthesis of ab initio and comparative gene prediction algorithms. *Genome Biol* 7: R24.

Liu C, Pitts RJ, Bohbot JD, Jones PL, Wang G, and Zwiebel LJ (2010) Distinct olfactory signaling mechanisms in the malaria vector mosquito *Anopheles gambiae*. *PLoS Biol* 8.

Lu T, Qiu YT, Wang G, Kwon JY, Rutzler M, Kwon HW, Pitts RJ, van Loon JJ, Takken W, Carlson JR, and Zwiebel LJ (2007) Odor coding in the maxillary palp of the malaria vector mosquito *Anopheles gambiae*. *Curr Biol* 17: 1533-1544.

Maibeche-Coisne M, Jacquin-Joly E, Francois MC, and Nagnan-Le Meillour P (2002) cDNA cloning of biotransformation enzymes belonging to the cytochrome P450 family in the antennae of the noctuid moth *Mamestra brassicae*. *Insect Mol Biol* 11: 273-281.

Marinotti O, Nguyen QK, Calvo E, James AA, and Ribeiro JM (2005) Microarray analysis of genes showing variable expression following a blood meal in *Anopheles gambiae*. *Insect Mol Biol* 14: 365-373.

Marinotti O, Calvo E, Nguyen QK, Dissanayake S, Ribeiro JM, and James A (2006) Genome-wide analysis of gene expression in adult *Anopheles gambiae*. *Insect Mol Biol* 15: 1-12.

McIver SB (1982) Sensilla mosquitoes (Diptera: Culicidae). *J Med Entomol* 19: 489-535.

Meola SM, Sittertz-Bhatkar H, Pendleton MW, Meola RW, Knight WP, and Olson J (2000) Ultrastructural analysis of neurosecretory cells in the antennae of the mosquito, *Culex salinarius* (Diptera: Culicidae). *J Mol Neurosci* 14: 17-25.

Meola SM and Sittertz-Bhatkar H (2002) Neuroendocrine modulation of olfactory sensory neuron signal reception via axo-dendritic synapses in the antennae of the mosquito, *Aedes aegypti*. *J Mol Neurosci* 18: 239-245.

Mortazavi A, Williams BA, McCue K, Schaeffer L, and Wold B (2008) Mapping and quantifying mammalian transcriptomes by RNA-Seq. *Nat Methods* 5: 621-628.

Neira-Oviedo M, Tsyganov-Bodounov A, Lycett GJ, Kokoza V, Raikhel AS, and Krzywinski J (2010) The RNA-Seq approach to studying the expression of mosquito mitochondrial genes. *Insect Mol Biol*.

Pennetier C, Warren B, Dabire KR, Russell IJ, and Gibson G (2010) "Singing on the wing" as a mechanism for species recognition in the malarial mosquito *Anopheles gambiae*. *Curr Biol* 20: 131-136.

Pitts RJ, Fox AN, and Zwiebel LJ (2004) A highly conserved candidate chemoreceptor expressed in both olfactory and gustatory tissues in the malaria vector *Anopheles gambiae*. *Proc Natl Acad Sci U S A* 101: 5058-5063.

Pitts RJ and Zwiebel LJ (2006) Antennal sensilla of two female anopheline sibling species with differing host ranges. *Malar J* 5: 26.

Qiu YT, Smallegange RC, Hoppe S, van Loon JJ, Bakker EJ, and Takken W (2004) Behavioural and electrophysiological responses of the malaria mosquito *Anopheles gambiae* Giles sensu stricto (Diptera: Culicidae) to human skin emanations. *Med Vet Entomol* 18: 429-438.

Qiu YT, van Loon JJA, Takken W, Meijerink J, and Smid HM (2006) Olfactory Coding in Antennal Neurons of the Malaria Mosquito, *Anopheles gambiae. Chem Senses* 31: 845-63
Robertson HM and Kent LB (2009) Evolution of the gene lineage encoding the carbon dioxide receptor in insects. *J Insect Sci* 9: 19.

Robinson MD and Oshlack A (2010) A scaling normalization method for differential expression analysis of RNA-seq data. *Genome Biol* 11: R25.

Rogers ME, Jani MK, and Vogt RG (1999) An olfactory-specific glutathione-S-transferase in the sphinx moth *Manduca sexta*. *J Exp Biol* 202: 1625-1637.

Sarov-Blat L, So WV, Liu L, and Rosbash M (2000) The *Drosophila takeout* gene is a novel molecular link between circadian rhythms and feeding behavior. *Cell* 101: 647-656.

Sato K and Touhara K (2009) Insect olfaction: receptors, signal transduction, and behavior. *Probl Cell Differ* 47: 121-138.

Schymura D, Forstner M, Schultze A, Krober T, Swevers L, Iatrou K, and Krieger J (2010) Antennal expression pattern of two olfactory receptors and an odorant binding protein implicated in host odor detection by the malaria vector *Anopheles gambiae*. *Int J Biol Sci* 6: 614-626.

Siju KP, Hansson BS, and Ignell R (2008) Immunocytochemical localization of serotonin in the central and peripheral chemosensory system of mosquitoes. *Arthropod Struct Dev* 37: 248-259.

Smallegange, RC, Qiu YT, van Loon JJA, and Takken W (2005) Synergism between ammonia, lactic acid and carboxylic acids as kairomones in the host-seeking behaviour of the malaria mosquito

Anopheles gambiae sensu stricto (Diptera: Culicidae). *Chem Senses* 30: 145-52.

Sutcliffe JF (1994) Sensory bases of attractancy: morphology of mosquito olfactory sensilla - a review. *J Am Mosq Control Assoc* 10: 309-315.

Takken W and Knols BG (1999) Odor-mediated behavior of Afrotropical malaria mosquitoes. *Annu Rev Entomol* 44: 131-157.

t' Hoen PA, Ariyurek Y, Thygesen HH, Vreugdenhil E, Vossen RH, de Menezes RX, Boer JM, van Ommen GJ, and den Dunnen JT (2008) Deep sequencing-based expression analysis shows major advances in robustness, resolution and inter-lab portability over five microarray platforms. *Nucleic Acids Res* 36: e141.

Tichy AL, Ray A, and Carlson JR (2008) A new *Drosophila* POU gene, *pdm3*, acts in odor receptor expression and axon targeting of olfactory neurons. *J Neurosci* 28: 7121-7129.

Touhara K and Vosshall LB (2009) Sensing odorants and pheromones with chemosensory receptors. *Annu Rev Physiol* 71: 307-332.

Tremblay P-L and Hallenbeck PC (2009) Of blood, brains and bacteria, the Amt/Rh transporter family: emerging role of Amt as a unique microbial sensor. *Molec Micro* 71: 12-22.

Verhulst NO, Takken W, Dicke M, Schraa G, and Smallegange RC (2010) Chemical ecology of interactions between human skin microbiota and mosquitoes. *FEMS Microbiol Ecol* 74: 1-9.

Vogt RG and Riddiford LM (1981) Pheromone binding and inactivation by moth antennae. *Nature* 293: 161-163.

Wang G, Carey AF, Carlson JR, and Zwiebel LJ (2010) Molecular basis of odor coding in the malaria vector mosquito *Anopheles gambiae*. *Proc Natl Acad Sci U S A* 107: 4418-4423.

Wang Z, Gerstein M, and Snyder M (2009) RNA-Seq: a revolutionary tool for transcriptomics. *Nat Rev Genet* 10: 57-63.

Warr E, Aguilar R, Dong Y, Mahairaki V, and Dimopoulos G (2007) Spatial and sex-specific dissection of the *Anopheles gambiae* midgut transcriptome. *BMC Genomics* 8: 37.

Xia Y, Wang G, Buscariollo D, Pitts RJ, Wenger H, and Zwiebel LJ (2008) The molecular and cellular basis of olfactory-driven behavior in *Anopheles gambiae* larvae. *Proc Natl Acad Sci U S A* 105: 6433-6438.

Xu PX, Zwiebel LJ, and Smith DP (2003) Identification of a distinct family of genes encoding atypical odorant-binding proteins in the malaria vector mosquito, *Anopheles gambiae*. *Insect Mol Biol* 12: 549-560.

Zwiebel LJ and Takken W (2004) Olfactory regulation of mosquito-host interactions. *Insect Biochem Mol Biol* 34: 645-652.

CHAPTER IV

OBSERVATIONS ON THE BIONOMICS OF M FORM *ANOPHELES GAMBIAE* FROM A RICE GROWING AREA OF GHANA.

Preface

The following publication by Charlwood et al. has been reviewed by *Medical and Veterinary Entomology* and is under revision. I was a listed author on this publication based on my contributions to the work performed in Okyereko, Ghana in late June and early July 2010. My direct contributions in the field included mapping the rice fields, assisting in mosquito collections, and observing mating swarms. I returned from the study site with several thousand frozen mosquitoes, collected from various sites and at various developmental stages. I sampled from those collections to determine the exact *An. gambiae* sub-species and molecular forms (M vs. S) by using single fly DNA isolations and diagnostic PCRs. Additionally, I contributed to the data analysis, writing, and editing of the manuscript. Most notably I drafted the final section of the discussion, which suggests the use of intermittent irrigation as a possible mosquito control measure in the village. The idea was not original, as Derek Charlwood first introduced the topic to me while in the village. However, I subsequently researched the method in order to inform my own opinion about its potential utility in Okyereko. I intend to return to the village in

2011 to continue this research and to discuss intermittent irrigation in more detail with the village elders and local farmers.

Introduction

Mosquito survival is an important parameter affecting the transmission of malaria since even in species considered 'good' vectors only a small proportion of the population survives through the extrinsic cycle of the parasite. In many instances survival rates per cycle of malaria vectors are similar (Hii et al., 1990) and may be higher than non-vectors (Gillies and De Meillon, 1968). It used to be thought that mortality in mosquitoes was independent of age, and that, as such, dividing the population into nulliparous (insects which have never laid eggs) and parous (those which have) classes was sufficient to determine survival rates if the time taken to complete the first cycle was known. This is because Anophelines are generally gonotrophically concordant, at least after the first gonotrophic cycle, and a single blood meal is usually sufficient to complete egg development. More detailed data has generally indicated, however, that survival is age dependent and that parous rate data by itself is not an especially useful measure (Clements and Paterson, 1981). Survival is linked to the activities performed in each oviposition cycle, some of which are more hazardous than others, rather than the time taken to complete them, hence survival per cycle is more important than survival per day (Burkot et al., 1990).

For estimating vectorial capacity, however, an estimate of calendar age may be more important. Calendar age depends, to a certain extent, on the feeding frequency of the mosquito, which itself depends, in part, on the time taken by the mosquito to return to feed following oviposition. In tropical conditions a mosquito which returns to feed immediately after oviposition, is likely to have a two-day feeding frequency and will go through five gonotrophic cycles (with all their associated hazards) before being a possible vector; one with a delay between oviposition and re-feeding has a three-day frequency and only need complete three cycles before becoming a vector. Factors affecting post-oviposition behaviour in malaria vectors, however, remain poorly studied or understood. Different species may have inherently different behaviours, a delay being perhaps obligatory in some species but rarely occurring in others (e.g. *Anopheles triannulatus* and *Chagasia bonnea* in Brazil, Charlwood and Wilkes 1981; Wilkes and Charlwood, 1979). In practice no population is likely to consist exclusively of one or other kind of mosquito and environmental factors may determine post-oviposition behaviour as much as, or more than, inherent behavioural or physiological ones. For example, both *An. farauti* from Papua New Guinea and *An. funestus* from Mozambique return to feed more rapidly in the presence of moonlight (Charlwood et al, 1986; Birley and Charlwood, 1989; Kampango et al., 2010). The time taken to return to feed can be determined by examination of the female's

reproductive system, those that return to feed shortly after oviposition having large ovariolar sacs that contract over a 24hr period.

The behaviour of vectors from emergence to their first oviposition is also critical for malaria transmission since mosquitoes that acquire an infection during their first oviposition cycle are more likely to survive and transmit the disease than those that become infected during later cycles. In many instances some or all of the emerging population go through a 'pre-gravid' phase and require two blood-meals to complete the first cycle (Gillies 1953, 1956; Charlwood et al., 1997, 2003). The number of insects that do this can also be determined by the examination of the ovaries. Between emergence and oviposition, in addition to taking an eventual blood-meal sufficient for maturation of the ovaries, the mosquitoes need to mate. This only needs to be done during this first cycle. Males of *Anopheles gambiae* s.l., a principal malaria vector group in Africa, deposit a gelatinous 'plug' in the females' common oviduct at the time of insemination (Gillies, 1956). This is easily seen upon dissection. The plug is absorbed over the following 24 hrs. The behaviour of females during their first oviposition cycle can therefore be determined by examination of their ovaries. This was therefore done with a population of *An. gambiae* from a rice growing area in Ghana.

Methods

Description of Study Site

The Okyereko Irrigation Project (5o 24.87' N 0o 36.25 W), some 70 km to the west of Accra, was constructed in 1974 for sustainable rice agriculture (Okoye et al., 2005; Dzodzomenyo and Simonsen 1999). The village of Okyereko consists of 80 relatively rundown cement houses, circa 5km from the coast and is bordered on two sides by extensive irrigated rice fields (Figure 1). The collection and dissection methods used were the same as those described from a study in the village in 2009 (Charlwood et al. 2011). In particular a Furvela tent-trap with two hosts inside was run for 23 consecutive nights in the middle of the village. The trap was similar to that described by Govella et al. (2009) with the slight difference that wire instead of string was used to support the trap away from the edge of the opening in the tent. Since the collection periods were similar in both years a comparison of population growth rates and estimates of mosquito survival was possible. Ad hoc searches for resting mosquitoes were undertaken in 7 houses and kitchens scattered throughout the village.

Dissection

Mosquitoes were dissected according to the schedule described by Charlwood et al. (2011, in press). Briefly insects were divided into the following categories: ***Virgin*** with ovarioles at Stage I, spermatheca empty; ***Plug unfed*** sperm in the spermatheca with a mating plug (probably the same calendar age as virgins (Charlwood et al., 2003); ***Nulliparous***

Stage I with ovarioles at Stage I with sperm in the spermatheca but without a mating plug; ***Nulliparous Stage II*** with sperm in the spermatheca in which yolk was present in the terminal ovariole; ***Parous with sacs*** with a sac with some distension still present – these mosquitoes were considered to have oviposited on the night that they were collected; ***Parous without sacs*** in which the sac from the previous ovipostion had contracted and in which, therefore, there had been a delay between oviposition and returning to feed.

Together these enabled a number of measurements to be made. Specifically, the sac/no-sac ratio provides an estimate of the mean oviposition cycle duration in parous insects and hence the number of oviposition cycles needed to complete the extrinsic cycle of the malaria parasite and the proportion of the parous population being sampled. The virgin/plug ratio provides an estimate of mating success in newly emerged females. The number of insects feeding with undeveloped ovaries provides an estimate of the pre-gravid rate in first feeding insects. The parous rate can be used to estimate survival using time-series analysis if certain conditions are met.

On four occasion's mosquitoes that were dead on arrival at the laboratory and those that were alive were dissected separately. In order to dissect dead mosquitoes the abdomen was punctured with needles so that the saline solution entered and re-hydrated the internal organs. After

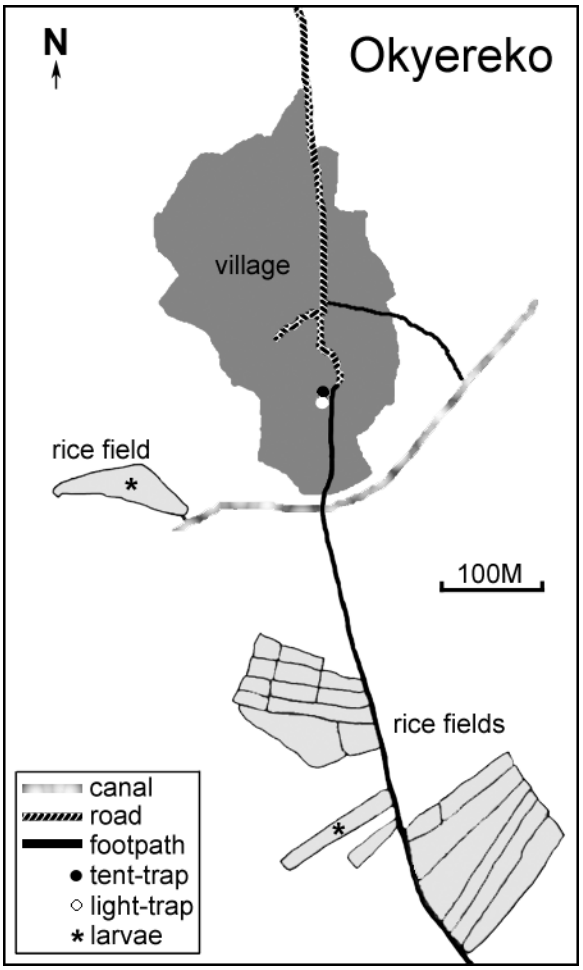


Figure. 1. Map of Okyereko village showing the location of sample sites and a selection of fields. The other fields to the south, east and west of the village were not mapped. The rice paddies observed to contain large numbers of *Anopheles gambiae* larvae at the start and end of the study are indicated by a star. Map created by D. Charlwood and J. Pitts using hand-held GPS unit (Charlwood et al. 2011).

being left for 10 or 15 minutes they could, with care, be dissected in the usual manner.

Selected samples were preserved in a solution of RNA Later (Ambion Inc.) + Triton X-100 (0.1% V/V). These were stored at 4 degrees centigrade for the duration of the field collection period then frozen at -20 degrees centigrade until removal for PCR analysis. Single fly PCRs were performed as follows: Heads of selected individuals were removed. Carcasses were crushed in 25ul of a buffered solution (10mM Tris pH8.2, 1mM EDTA, 25mM NaCl, 200ug/ml proteinase K) and incubated at 37 degrees for 20 minutes. Samples were heated to 95 degrees for 2 minutes to inactivate proteinase K and centrifuged briefly at 4 degrees. 1ul of each sample was used as a PCR template to determine M or S molecular form using the S200 X6.1 method as described (Santolamazza et al., 2008). Individual samples displaying the S-form PCR amplicon were subjected to subsequent PCR analysis to clarify *An. gambiae* complex species as described (Scott et al., 1993). The presence of circumsporozoite (CS) antigens of *P. falciparum* was determined using the sandwich Enzyme-Linked Immunosorbent Assay (ELISA) using the protocols of Wirtz (1987).

Environmental Monitoring

Air temperatures were measured with a digital logger (Tiny-tag) that recorded every half hour with the exception of a number of evenings when temperatures were recorded every minute.

Data Analysis

Survival rate estimation by time-series analysis according to the methods of Birley and colleagues (Charlwood et al., 1985; Holmes and Birley, 1987; Hii et al., 1990; Mutero and Birley, 1989) were applied to the data as were estimates of Garrett-Jones and Grab (1964), which depends on knowing the relative feeding frequencies of the different aged mosquitoes. These were determined by determining cross-correlation lag lengths between successive age groups using the program MINITAB. Following Holmes and Birley (1987) cross-correlations (R) were considered to be significant if R=2/sqrt(d). Where d = the number of observations (in our case 23) = 0.416. The Simpson index of species diversity (1-D), which is sensitive to changes in the more abundant species, was obtained for both tent and light-trap using the formulae described by Krebs (1999) where (1- D) = 1-Σp2i where pi. = proportion of species in the community. The index ranges from 0 (low diversity) to almost 1 (1-1/number of species in the sample).

Results

Anopheles gambiae s.l. was by far the most common mosquito collected (Table 1). As was the case in 2009 (Charlwood et al., 2011) the great majority of those identified to species and form were M form *An. gambiae*. However, a larger proportion of the individuals from 2010 were

of the S form (Table 2). Of 107 *An. gambiae* females identified to form from the light trap, 17 were of the S form (16%). Similarly, 10 out of 116 (9%) examined from the tent trap were S form. No statistical difference in the collected samples was observed (Fisher's exact test, p=0.105), such that the combined sample was 12% S (27 S, 196 M). A sample of *An. gambiae* collected from puddles in a fallow rice field showed a similar composition, being 8% S form (6 S, 74 M), while a sample collected from a puddle in the village was composed entirely of M form individuals (n=48). Moreover, of the individuals collected by sweep net from swarms or as mating pairs and identified to molecular form, all were of the M form (110 males, 8 females). The latter result demonstrates that the M form was mating within the village itself, while swarming sites for the S form remained undiscovered. Twice the proportion of blood-fed and gravid females was caught in the light-trap compared to the tent-trap but in neither trap was the other possible vector, *An. funestus* Giles common, a total of 44 being caught during the whole study period.

The most severe flooding of the previous 19 years occurred on the days following a day of heavy rain on the 21st of June. This inundated much of the surrounding area and many of the rice fields close to the village. Following this rain, however, with the exception of a single evening, when there was sufficient wind to disrupt swarming, conditions were uniformly calm. Indoor mean temperatures from 29 June - 3 July were

	Anopheles					***Culex***		***Mansonia***		***Aedes***
	gam. M	*gam.* S	*fun.*	*phar.*	*zie.*	*quin.*	*tri.*	*afr.*	*uni.*	*aeg.*
Tent	11491	1567	34	139	5	961	178	20	118	5
Light	937	127	10	4	0	16	4	15	17	0

Table 1. Numbers of the different species collected in the tent-trap (*n* = 24) and light-trap (*n*= 8). Abbreviations: (*gam.* M) – *Anopheles gambiae* M form; (*gam.* S) – *Anopheles gambiae* S form; (*phar.*) – *Anopheles pharoensis*; (*zie.*) – *Anopheles ziemanni*; (*quin.*) – *Culex quinquefasciatus*; (*tri.*) – *Culex tritaeniorhynchus*; (*afr.*) – *Mansonia africana*; (*uni.*) – *Mansonia uniformis*; (*aeg.*) – *Aedes aegypti*.

	tent	light	field	village	swarms
M	106	90	74	48	118
S	10	17	6	0	0

Table 2. Molecular forms of *Anopheles gambiae* collected by various methods.

27.14 ± 2.13 oC and outdoors from the 3rd -12 July 26.63 ± 3.66 oC. Hence the observed changes in the population reflect rates under apparently 'ideal' characteristics rather than merely responses to environmental perturbation, as was the case in 2009 (Charlwood et al., *in press*). Figure 2 shows the population changes observed from the tent-trap collections. Despite the possible elimination of many potential breeding sites due to the flooding populations of *An. gambiae* and *An. pharoensis* increased during the study while those of *Cx. tritaeniorhynchus* and *Cx. quinquefasciatus* remained more or less stable. While the population of *An. pharoensis* continued to increase, *An. gambiae* declined in the last three days of collection. The increase of *An. pharoensis* was best described by an exponential function while that of the *An. gambiae*, excluding the final decline, by a linear function. Despite being a growing population some of the *An. pharoensis* were multi-parous (in fewer than 15 mosquitoes dissected two insects were 3-parous and one was 5-parous). The Simpson's index (1-D) was 0.357 from the tent-trap and 0.299 from the light-trap (calculated treating the 12% of the *An. gambiae* S form as a separate species to the M form). Thus the tent-trap collected a more diverse population of mosquitoes than the light-trap.

1787 *An. gambiae* females were dissected. Total numbers of the different age groups dissected by trapping method are shown in Table 3.

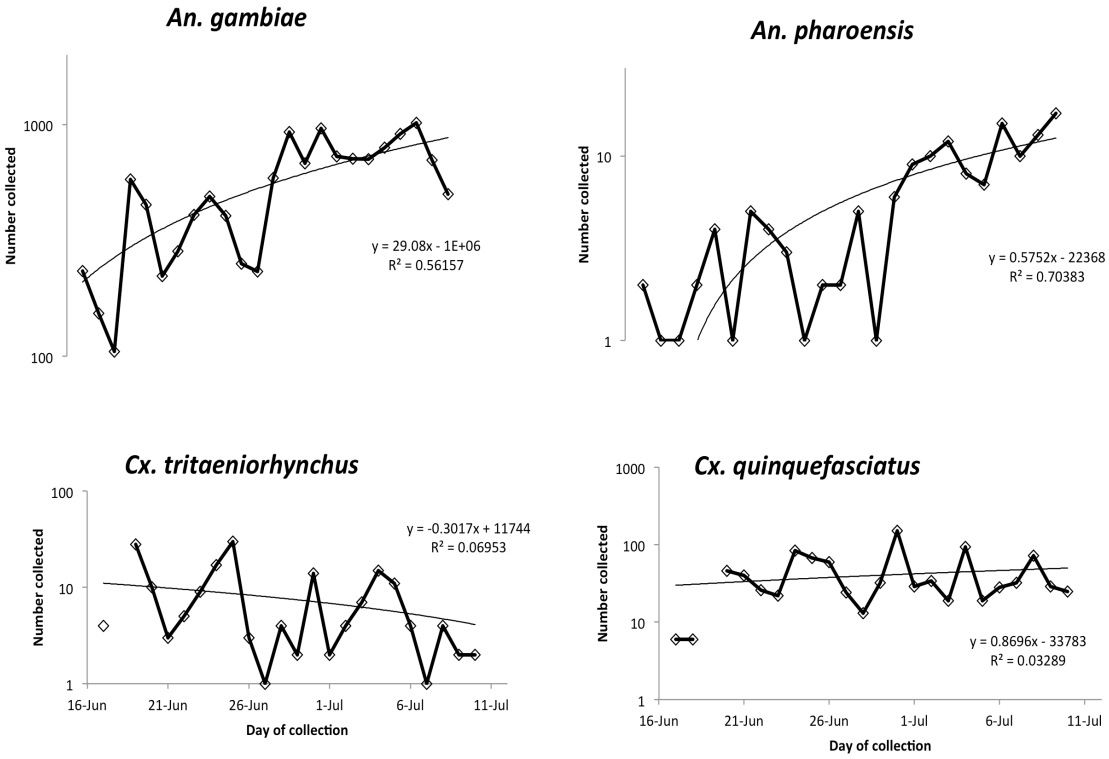


Figure 2. Numbers of *Anopheles gambiae*, *An. pharoensis*, *Culex tritaeniorhynchus* and *Cx. quinquefasciatus* collected in a 'Furvela' tent-trap from the village of Okyereko, Ghana, June-July 2010.

The age structure among the *An. gambiae* dissected was similar from the two types of trap (Chi-square light and tent trap in this group = 1.161, p = 0.281). On four days of collection live and dead insects from the tent-trap were sorted and dissected separately. 2119 (68%) of the 3133 unfed females collected at these times were alive at the time of collection. These proportions were similar for the other abdominal stages of *An. gambiae* and for *Cx. quinquefasciatus*. A greater proportion of the *An. pharoensis* (0.625) had, however, died before collection (Chi-square 54.01 p = 0.0001). With the exception of an excess number of N I's among the live *An. gambiae* (Fishers two tail test comparing (virgin+ plug) and N I's, p = 0.0431) the age of the 166 dead females dissected was similar to the 206 dissected that were alive on collection (Table 4). Following the initial light-trap collections samples were restricted to the sentinel tent trap. The rate of increase of the *An. gambiae* was lower than that observed in 2009. This was in part due to a drop in the numbers of parous mosquitoes in the population. Thus, the estimated growth rate of the pre-gravid population was higher than the other age groups (Figure 3). Given the major changes in density observed estimates of survival based on a stationary population are not, strictly, valid. Table 4 gives the correlation coefficients between the different age groups with up to six days lag. The correlation between parous and total population, with and without the correction for the numbers pre-gravid failed to produce any significant cross correlations

	virgin	plug	NI	NII	sac	no-sac
Light-trap	28	46	20	18	27	43
Tent-trap	241	388	233	163	218	362
Total	269	434	253	181	245	405

Table 3. Age structure of the *An. gambiae* dissected from Okyereko June –July 2010. (see Methods for explanation of categories).

	virgin	plug	NI	NII	sac	no-sac
dead	25	48	25	15	23	30
live	25	49	47	13	22	50
Total	50	97	72	28	45	80

Table 4. Age structure of *An. gambiae* that were dead or alive at the time of collection from the tent-trap, Okyereko, Ghana. (see Methods for explanation of categories).

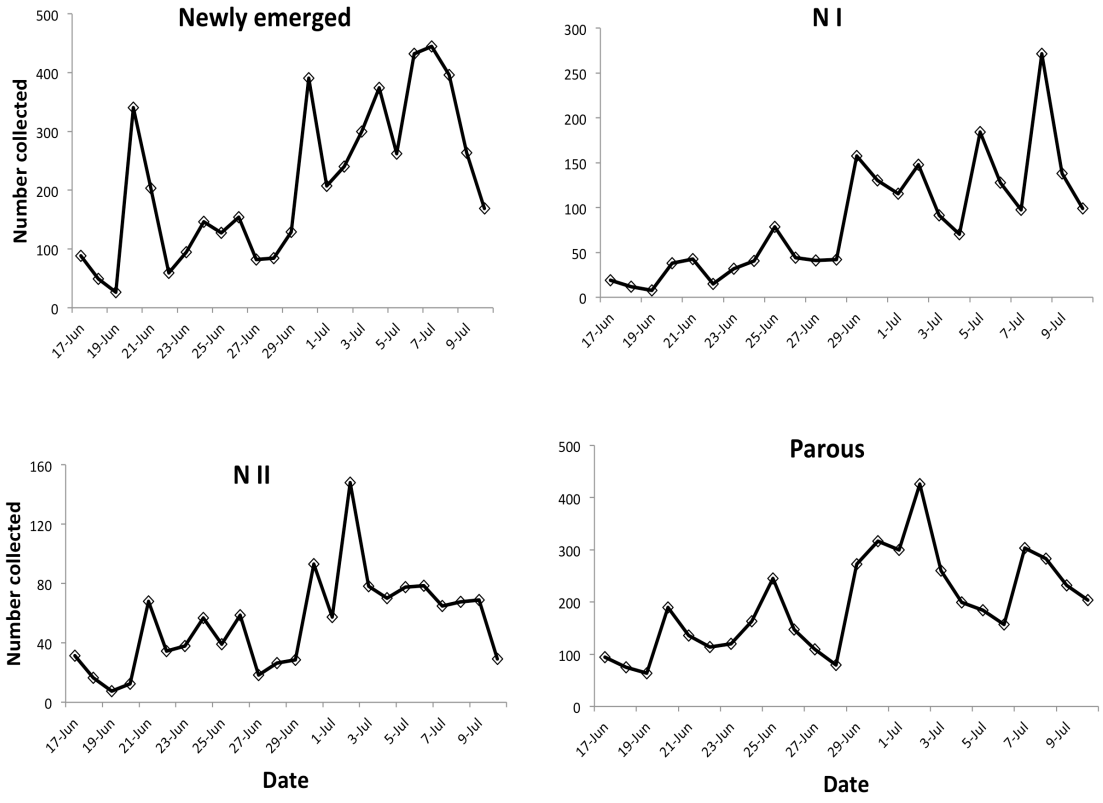


Figure 3. Estimated number of *Anopheles gambiae* by gonotrophic age collected in a 'Furvela' tent-trap from the village of Okyereko, Ghana, June-July 2010.

(beyond that of Day 0). Thus, time series methods (Holmes and Birley, 1987; Mutero and Birley 1989) cannot be applied to the data to estimate survival.

The data does, however, enable an estimate of the feeding frequency to be obtained. Correlations between virgin and recently mated females were significant at a lag on one day while that between (virgin+plug) and NI was significant at one and two days. It was four days between (virgin+plug) and NII's and three between NI and NII's (Table 5). The estimated oviposition cycle duration of 2.65 ±0.17 days was the same for light-trap and tent-trap and did not differ among the live and dead mosquitoes dissected (2.55 ±0.18 days versus 2.71± 0.18 days, Chi-square p = 0.1972). These estimates indicate that the population of *An. gambiae* in Okyereko fed on days 2,4,7,10,13 after emergence and oviposited for the first time sometime between day 4 and day 7. In other words they followed cycle number 5 described by Garrett-Jones and Grab (1964). With such a feeding schedule and a parous rate of 0.36 the daily survival rate obtained from the published curve is 84%. The mosquitoes have to survive 13 or 16 days before they might transmit malaria. The shorter period assumes that the initial pre-gravid meal can be infectious. Under such circumstances (and assuming density independent survival) 10.4% of the mosquitoes will survive long enough to be vectors. If it is not infectious then only 7.3% will survive the minimum time to be possible vectors. Overall, 10% of those that acquire the parasite in any of their first

	Lag						
Comparison	**Day 0**	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**
Virgin (V) /Plug (P)	0.63	0.51*	0.29	0.32	0.16	0.20	0.06
V/(V +P)	0.84	0.54*	0.32	0.33	0.24	0.23	-0.001
(V+P)/ N I	0.60	0.60*	0.50*	0.24	0.25	0.32	0.21
N II/ (V+P+N I)	0.64	0.39	0.40	0.39	0.51*	0.41	0.38
N II/(V+P)	0.59	0.36	0.40	0.30	0.54*	0.39	0.35
N II/ N I	0.56	0.33	0.30	0.46*	0.30	0.33	0.38
(NI + NII)/(V+P)	0.67	0.56	0.39	0.32	0.45*	0.43	0.33
Sac/No-sac	0.40	0.50*	0.17	-0.04	-0.16	0.25	0.31
Parous/Total	0.92	0.62					
Parous/(Total –V-P)	0.79	0.76	0.71	0.31	0.09	0.11	0.12
Parous/ (NI + N II)	0.69	0.63	0.30	0.33	0.25	0.34	0.44
Parous/N II	0.72	0.58	0.37	0.28	0.09	0.15	0.15

Table 5. Cross correlations at different lags between age classes of *An. gambiae* from Okyereko village, June-July 2010. Asterisk (*) marks significant correlations according to the formula of Holmes and Birley (1987).

three cycles will survive to transmit. These proportions will be smaller if age specific mortality affects survival (Clements and Paterson, 1981).

With the exception of one windy night the mean proportion of the most recently emerged insects (i.e. virgin and plug) with a mating plug was 0.63 ± 0.04. (Figure 4). Following the one evening that windy conditions at sunset prevented the males from swarming over these markers only three of the nine newly emerged insects collected the following morning had mating plugs. Although not quite significantly different from the expected proportion (Fishers exact test p = 0.094) this was the lowest proportion observed of any night (four times this number would normally have been collected). The following day18 of 21 newly emerged insects had mating plugs. This was the highest proportion (0.86) observed on any night and implies that most mating takes place (in swarms) at dusk and that failure to mate on one night may not incur a high mortality cost. 2933 mosquitoes from 10 days of collection were analyzed for the presence of circumsporozoite protein. Eighteen (0.61% s.d. ± 0.49%) were positive. The rate among tested insects varied considerably from day to day, six of the ten days not having any positive insects. Among the estimated 1435 parous mosquitoes included in the samples tested by ELISA the overall mean sporozoite rate was 1.25%, similar to that obtained in 2009.

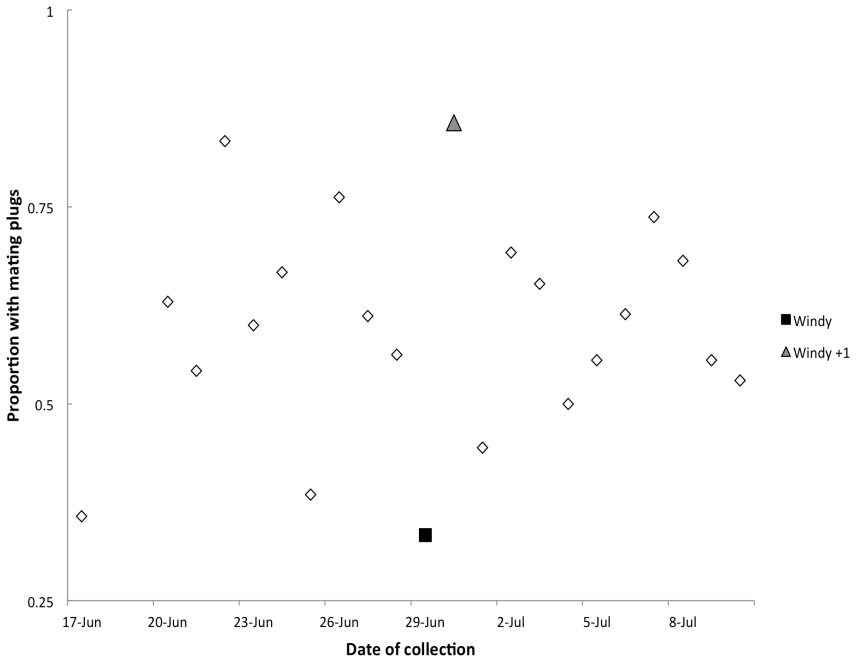


Figure 4. The proportion of newly emerged *Anopheles gambiae* with mating plugs, by date of collection, Okyereko, Ghana, June-July 2010. The black square denotes the collection following an evening when wind disrupted swarming behaviour and the grey triangle the night after that.

Discussion

Despite collecting mosquitoes in a slightly more central location in the village, compared to the collection sites of 2009, there was still a great preponderance of pre-gravid 'first-feeding' insects in the collections. While time-series estimates failed to produce an appropriate estimate of survival and prevented a sensible estimate to be determined the data enabled estimates of the feeding and oviposition rhythms to be made which together with the parous rate enabled an estimate of daily survival to be made using the methods of Garrett-Jones and Grab (1964). Notwithstanding the caveat that the population was not stationary, the estimated daily survival rate of 84% is similar to estimates obtained for *An. arabiensis* and S form *An. gambiae* from Tanzania (Gillies and Wilkes, 1965;Charlwood et al., 1995). At the highest observed rate of transmission one in a hundred mosquitoes (or up to 10 per night) was a vector of malaria and, despite the 'paddy paradox' (Ijumba and Lindsay, 2001), the disease remains a problem in Okyereko.

At the lowest densities the proportion of new recruits in the population was 30%. The increase to 70% observed at the highest densities was in part due to a decline in the number of parous insects in the population rather than a proportionate increase in new recruits. In other areas mortality is extremely high among larvae (Charlwood, 2003; Service, 1977). For the population to suffer the extreme 'mortality'

observed in newly recruited adults would seem to be a double blow. It may be that in a population like Okyerko survival through to the adult stage is high and that the loss among new recruits to the adult population is the larval mortality carried through to the adult stage. Housing in Okyereko is not conducive to mosquito resting and ad hoc collections produced very few insects. It is also possible that outdoor resting is costly, particularly for the new recruits.

It is perhaps not an accident that the populations that failed to be useful for time-series analysis have all been high density ones (Mutero and Birley, 1989; Charlwood JD and Smith TR unpublished observations, 1994). There are a number of reasons why even under the ideal environmental conditions experienced during the latter part of the study the time series methods might not have worked. One is that the dissections were inaccurate. There are two categories where decisions in classification might be biased since they are continuous rather than discrete conditions. These are the classification into sac or no-sac and the separation into mated Nulliparous insects with ovaries at Stage I or II. While errors in the former do not affect survival estimates (since both sets are parous), that of the latter affects estimates of numbers in the pre-gravid and Nulliparous categories. Nevertheless, summing the different categories and considering the NI population to be either gonotrophically inactive (pre-gravid) or gonotrophically active did not improve estimates. The relatively smooth increase in estimated numbers of pre-gravids in the

population implies that the dissections were relatively accurate, as do the 'sensible' lags at which peak correlations occurred in young age groups (and even between the sac and no-sac population). Although for much of the study the population increased regularly this increase was as well described linearly as exponentially (there being no difference in the r2). Although starting from a much smaller base the rise in numbers of the *An. pharoensis* was exponential. Although the number of *An. pharoensis* dissected was too small for survival rate estimation the observation of several multi-parous insects implies that this species could be a potentially important vector in Okyereko. Interestingly in their study of filariasis from the village Dzodzomenyo et al., (1999) found that despite *An. gambiae* being the main vector two of three specimens of *An. pharoensis* examined were infected, one of which was infectious. The rise in the *An. gambiae* population was lower than that observed in *An. arabiensis* from Tanzania (Charlwood 2003; Takken et al, 1995) but was higher in the new recruits than in the population as a whole; estimated numbers of parous mosquitoes actually going into a decline even as the number of new recruits was rising. Govella et al., (2009) report that the efficiency of both light-trap and Furvela tent-trap for S form *An. gambiae* is density dependent. It is possible that other traps would have collected even more than the 1000 or so *An. gambiae* at peak times. A comparison between different tent-traps in an area like Okyereko might provide useful data.

Another factor that might affect the estimates is that one age group may belong to a more closed population than another. For example, the virgin and recently mated insects may disperse away from the area while the N I's may be immigrants. Their relative contribution to the parous population may differ. The history and future of the 'first-feeding' insects is, therefore, still difficult to determine, in particular the mosquitoes that had poorly developed ovaries, had mated but no longer had a mating plug. The plug lasts for approximately 24hrs (Gillies, 1956) thus N I insects had presumably mated more than 24hrs before being collected but, unlike the insects with a plug, had not taken a blood-meal immediately after mating. Had they mated at dawn the plug may have been absorbed by the time they were examined. Even in places such as São Tomé where swarms of *An. gambiae* have been recorded the activity at dawn was a fraction of that observed at dusk (Charlwood et al., 2002). So mating at dawn is not likely to be the cause. It is possible that the insects find it hard to obtain a blood meal the night they mate and so are basically the remnants of the previous nights 'plug-positive' cohort.

Alternatively virgins that have previously fed may mate and digest their initial bloodmeal before returning a day later as mated N I's. Egg development in mosquitoes is dependent on JH levels, which do not rise until Day 3 post-eclosion, and/or mating (Noriega, 2004). Virgin mosquitoes that take a blood meal are presumably very young. We do not, however, know whether this second meal leads to ovarian development or

not (i.e. whether they take two pre-gravid meals) and what affect this might have on both survival and vectorial capacity.

Swarms of M form male *An. gambiae* were seen within the middle of the village. Numbers of pairs observed leaving swarms was far too low to account for the amount of mating that must have been occurring, (as judged by the number of plug positive females in the collection) indicating that mating was taking place elsewhere. Where mating was taking place, however, remains unknown. Nevertheless most mating probably occurs in swarms at dusk since in the tent-trap collection on the night following the one evening sufficiently windy at dusk to prevent swarming was the collection with the lowest proportion of plug-positive mosquitoes among newly emerged insects indicating that mating had been curtailed on that night. The following day the proportion of newly emerged insects with plugs was higher than on any other day. Since the wind ceased shortly after dusk these results imply that mating only takes place at dusk.

Rice fields are often major breeding sites for Anophelines (Mwangangi et al., 2010) as appears to be the case in Okyereko. Dzodzomenyo et al., (1999) describe a substantial increase in numbers of *An. gambiae* when the fields of Okyereko are irrigated at the end of the dry season. Given the large numbers of new recruits to the population anti-adult control measures will produce very little perceptible change in the mosquito population biting people even though they may reduce malaria transmission. Environmental management techniques, such as

intermittent irrigation in well-leveled fields and a planned irrigation systems, as is Okyereko, offer alternatives to adulticide-based interventions and indeed can have a great effect on local mosquito populations (Keiser et al., 2005a, b). Frequent draining, drying and subsequent re-flooding of rice fields can greatly increase Anopheline larval mortality (Mutero et al., 2000) ultimately reducing the adult population, which can sometimes dramatically lower rates of malaria transmission (Keiser et al., 2005a,b). Another potential benefit of intermittent irrigation would be the reduction in local mosquito diversity (Mwangangi et al., 2010), which might affect both disease-transmitting and nuisance biting mosquitoes. Establishing intermittent irrigation may take several years, during which time side-by-side test fields would be alternatively managed either by intermittent or continuous irrigation, while mosquito breeding and rice yields are carefully monitored in each. The already intense interest among villagers for malaria-reducing interventions may improve the likelihood of adoption if it can be demonstrated that mosquito breeding is reduced and that rice yields are at least equivalent if not improved, which often occurs in intermittently irrigated fields (Mutero et al., 2000;Keiser et al., 2005a,b).

References

Birley MH and Charlwood JD (1989) The Effect of moonlight and other factors on the ovipositon cycle of malaria vectors in Madang, Papua New Guinea. *Annals of Tropical Medicine and Parasitology*, **8**, 415-422.

Burkot TR, Graves PM, Paru R, Battistuta D, Barnes A, and Saul A (1990) Variations in malaria transmission rates are not related to Anopheline survivorship per feeding cycle. *American Journal of Tropical Medicine and Hygiene*, **43**, 321-7.

Charlwood JD, Tomás EVE, Salgueiro P, Egyir-Yawson A, Pitts RJ, and Pinto J (2011) Studies on the behaviour of peridomestic and endophagic M form *Anopheles gambiae* from a rice growing area of Ghana. *Bulletin of Entomol Res* March 15 [Epub ahead of print].

Charlwood JD, Billingsley PF, Takken W, Lyimo EOK, Smith T, and Meuwissen JME (1997) Survival and infection probabilities of anthropophagic Anophelines from an area of high prevalence of *Plasmodium falciparum* in humans. *Bulletin of Entomological Research***, 87**, 445-453.

Charlwood JD, Birley MH, Dagoro H, Paru R, and Holmes PR (1985) Assessing survival rate of *Anopheles farauti* (Diptera: Culicidae) from Papua New Guinea. *Journal of Animal Ecology*, **54**, 1003-1016.

Charlwood JD, Pinto J, Sousa CA, Ferreira C, Petrarca V, and do Rosário VE (2003) A mate or a meal' - Pre-gravid behaviour of female *Anopheles gambiae* from the islands of São Tomé and Príncipe, West Africa. *Malaria Journal,* **2**,7.

Charlwood JD, Paru MH, Dagaro H, and Lagog M (1986) The influence of moonlight and gonotrophic age on the biting activity of *Anopheles farauti* (Diptera, Culicidae) from Papua New Guinea. *Bulletin of Entomological Research,* **76**, 211-227.

Charlwood JD, Kihonda J, Sama S, Billingsley PF, Hadji H, Verhave JP, Lyimo E, Luttikhuizen PC, and Smith T (1995) The rise and fall of *Anopheles arabiensis* (Diptera, Culicidae) in a Tanzanian village. *Bulletin of Entomological Research*, **85**, 37-44.

Charlwood JD, Pinto J, Sousa CA, Ferreira C, and do Rosário VE (2002) The swarming and mating behaviour of *Anopheles gambiae* (Diptera: Culicidae) from São Tomé Island. *Journal of Vector Ecology*, **27**, 178-183.

Charlwood, JD (2003) **May the force be with you: measuring mosquito fitness in the field**. Chapter 5, In *Proceedings of a workshop on transgenic mosquitoes (Edited by Scott T and Takken W) Wageningen, The Netherlands.*

Charlwood JD and Wilkes TJ (1981) Observations on the biting activity of *Anopheles triannulatus bachmanni* from the Mato Grosso, Brazil. *Acta*

Amazonica, **11**, 411-413.

Clements AN and Paterson GD (1981) The analysis of mortality and survival rates in wild populations of mosquitoes. *Journal of Applied Ecology*, **18**, 373-399.

Dzodzomenyo M, Dunyo SK, Ahorlu CK, Coker WZ, Appawu MA, Pedresen EM. and Simonsen PE (1999) Bancroftian filariasis in an irrigation project community in southern Ghana. *Tropical Medicine and International Health*, **4**, 13-18.

Garrett-Jones C and Grab B (1964) The assessment of insecticidal impact on the malaria mosquito's vectorial capacity, from data on the proportion of parous females. *Bulletin of the World Health Organization,* **31**, 71-86.

Gillies MT (1953) The duration of the gonothrophic cycle in *Anopheles gambiae* and *An. funestus*, with a note on the efficiency on the hand catches. *East African Medical Journal,* **30**, 129-135.

Gillies MT (1956) A new character for the recognition of nulliparous females of *Anopheles gambiae*. *Bulletin of the World Health Organization,* **15**, 451-459.

Gillies MT and De Mellion B (1968) **The Anophelinae of Africa south of the Sahara (Ethiopian zoogeographical region)**. 2nd Edition. Publications of the South African Institute for Medical Reasearch N.º 54. Johannesburg, South Africa.

Gillies MT and Wilkes T (1965) A study of age composition of population of *Anopheles gambiae* Giles and *Anopheles funestus* Giles in North-eastern Tanzania. *Bulletin of Entomological Research,* **56**, 129-135.

Govella NJ, Chaki PP, Geissbuehler Y, Kannady K, Okumu F, Charlwood JD, Anderson RA. and Killeen GF (2009) A new tent trap for sampling exophagic and endophagic members of the *An. gambiae* complex. *Malaria Journal*, **8**: 1.

Hii JL, Birley MH, and Sang VY (1990) Estimation of survival rate and oviposition interval of *Anopheles balabacensis* mosquitoes from mark-recapture experiments in Sabah, Malaysia. *Medical and Veterinary Entomology*, **4**, 135-140.

Holmes PR and Birley MH (1987) An improved method for survival rate analysis from time series of haematophagous Dipteran populations. *Journal of Animal Ecology,* **56**, 427-440.

Ijumba JN and Lindsay SW (2001) Impact of irrigation on malaria in Africa: Paddies paradox. *Medical and Veterinary Entomology*, **15**, 1-11.

Keiser J, Singer BH, and Utzinger J (2005) Reducing the burden of malaria in different eco-epidemiological settings with environmental management: a systematic review. *Lancet Infectious Diseases,* **5**, 695-708.

Kampango A, Cuamba N, and Charlwood JD (2010) Does moonlight influence the biting behaviour of Anopheles *funestus* (Diptera: Culicidae)? *Medical and Veterinary Entomology* Nov 14. doi: 10.1111/j.1365-2915.2010.00917

Keiser J, De Castro MC, Maltese MF, Bos R, Tanner M, Singer BH, and Utzinger J (2005) Effect of irrigation and large dams on the burden of malaria on a global and regional scale. *American Journal of Tropical Medicine and Hygiene*, **72**, 392-406.

Krebs CJ (1998) *Ecological Methodology* Addison, Wesley Longman, Inc (2nd Edition)

Mutero CM and Birley MH (1989) The effect of pre-gravid development on the estimation of mosquito survival rates. *Journal of Applied Entomology,* **107**, 96-101.

Mutero CM, Blank H, Konradsen F, and van der Hoek W (2000) Water management for controlling the breeding of Anopheles mosquitoes in rice irrigation schemes in Kenya. *Acta Tropica*, **76**, 253-263.

Mwangangi JM, Shililu J, Muturi EJ, Muriu S, Jacob B, Kabiru EW, Mbogo CM, Githure J, and Novak RJ (2010) Anopheles larval abundance and diversity in three rice agro-village complexes Mwea irrigation scheme, central Kenya. *Malaria Journal*, **9**, 228.

Noriega FG (2004) Nutritional regulation of JH synthesis: a mechanism to control reproductive maturation in mosquitoes? *Insect Biochemistry and Molecular Biology*, **34**, 687-693.

Okoye PN, Wilson MD, Boakye DA, and Brown CA (2005) Impact of the Okyereko irrigation project in Ghana on the risk of human malaria infection by Anopheles species (Diptera: Culicidae). *African Entomology*, **13**, 249-253.

Santolamazza F, Mancini E, Simard F, Qi Y, Tu Z, and della Torre A (2008) Insertion polymorphisms of SINE200 retrotransposons within speciation islands of *Anopheles gambiae* molecular forms. *Malaria Journal*, **7**,163.

Scott JA, Brogdon WG, and Collins FH (1993) Identification of single specimens of the *Anopheles gambiae* complex by the polymerase chain reaction. *American Journal of Tropical Medicine and Hygiene,* **49**, 520-529.

Service MW (1977) Mortalities of the immature stages of species B of the *Anopheles gambiae* complex in Kenya: comparison between rice fields and temporary pools, identification of predators, and effects of insecticidal spraying. *Journal of Medical Entomology*, **13**, 535-545.

Takken W, Charlwood JD, Billingsley PF, and Gort G (1998) Dispersal and survival of *Anopheles funestus* and *A. gambiae* s.l. (Diptera, Culicidae) during the rainy season in southeast Tanzania. *Bulletin of Entomological Research*, **88**, 561-566.

Wilkes TJ and Charlwood JD (1979) A rapid gonotrophic cycle in *Chagasia bonneae* from Brazil. *Mosquito News*, **39,** 137-139.

Wirtz R, Zavala F, Charoenvit Y, Campbell GH, Burkot TR, Schneider I, Esser KM, Beaudoin RL, and Andre RG (1987) Comparative testing of *Plasmodium falciparum* sporozoite monoclonal antibodies for ELISA development. *Bulletin of the World Health Organization,* **65**, 39-45.

CHAPTER V

SUMMARY AND FUTURE DIRECTIONS

Key Findings and Follow Up

By using the tools of molecular biology, behavior and computational biology, we have conducted studies in *An. gambiae* that offer new insights into the biology of one of the major malaria mosquitoes in sub-Saharan Africa. The identification *AgIrs*, a new class of chemoreceptors, adds significantly to our understanding of olfaction in *An. gambiae.* These genes are homologous to the *DmIrs* (Benton et al. 2009), which have proven roles in odor reception in coeloconic sensilla. Their expression is significantly enhanced in *An. gambiae* antennae and maxillary palps. Next we will need to determine whether *AgIrs* are expressed in grooved pegs. Furthermore, we will attempt to express *AgIrs* in heterologous systems to elucidate their chemoreceptive spectra. We speculate that *AgIrs* may be responsible for sensing important volatiles that *AgOrs* lack significant responses to, including amines like butylamine and ammonia or lactic acid that elicit grooved peg responses. The requirement of *AgIr76b* in larval behavioral responses to butylamine gives us an early target. Finally, it will be critical for us to be able to demonstrate in-vivo that *AgIrs* and *AgOrs* are required for odor responses, both at the physiological and behavioral levels. RNAi technology gives us an immediate opportunity to attempt to

transiently knock down chemoreceptor targets in adult stage mosquitoes for this purpose. We are also exploring transgenic technologies that are designed to stably knock down gene expression in the laboratory. I believe that making the leap from strong correlative data that link *An. gambiae* odor sensitivity with chemoreceptor expression and *AgOr* or *AgIr* function, will be an imperative that our field needs to meet in order to completely justify the potential for this line of research to impact disease transmission. We can no longer assume that these gene families are responsible for mediating olfactory signaling and must provide direct evidence for their necessity. Otherwise we risk spending a lot of time and resources pursuing pharmacological agents that target *AgOrs* or *AgIrs* that will have little or no effect on mosquito behavior. To that end we are currently working with mosquito behaviorists to address questions about modifications of host seeking using compounds identified in pharmacological screens.

The transcriptome profiles of the antennae and maxillary palps of female and male *An. gambiae* have also moved us in a new and exciting direction. Documenting more of the molecular components of *An. gambiae* chemosensory tissues that are like to mediate signal transduction is necessary if we hope to fully understand the process. To that end we have identified numerous gene families that are enhanced in chemosensory tissues, including many that have not been implicated in insect olfaction. For example, the ammonium transporters described in Chapter II may

participate in ammonia sensing. We have the tools at our disposal to study the localization patterns of the genes, examine their potential functions in heterologous expression systems, and to attempt to knock down their transcripts in live animals.

Not surprisingly, we uncovered similarities as well as differences in the gene expression profiles of antenna and maxillary palps and between females and males. The former result may give us insights into specific signaling mechanisms that are either shared or divergent between antennae and palps. The latter result may open up new comparative avenues of research that could determine differences in olfactory processes between the sexes. Importantly, very little research has focused on male mosquito chemosensory biology. Ultimately, more studies of male *An. gambiae* biology will be needed in order for us to appreciate the potential meaning of differential gene expression. For example, the higher overall expression of *AgOrs* in female antennae and palps could result in increased sensitivity to odors. This hypothesis is testable using electrophysiological and behavioral assays. In addition, we have observed enhanced expression of *AgOrs* in male bodies. We intend to explore the nature of this result, looking specifically for *AgOrs* in sperm, where they may mediate chemotaxis. We may also address the possibility of cryptic olfactory (perhaps pheromonal) function in other male appendages, like legs and wings. Ultimately, gene expression profiling is not an end unto itself, but is only a tool useful for identifying new genes of

interest. Those genes could become targets for novel olfactory-based interventions that interfere with mosquito-host interactions.

Although the work presented in Chapter IV was only indirectly related to *An. gambiae* olfaction, the experience was invaluable to me. The trip was my first to Africa and therefore was my first opportunity to observe *An. gambiae* in its natural environment. I got to spend quality time with excellent mosquito researchers, learning more about mosquito ecology. Moreover, I've established some important contacts that may lead to future collaborations. We have a long-standing interest in studying *AgOr* evolution, from the perspective that these genes are undergoing rapid evolution and that AgOr polymorphisms among local populations of *An. gambiae* may be relevant to endophily, anthropomorphism, or other other aspects of sensory biology. Having collaborators in the field could open up the possibility of gathering samples from many sites that we can use for this purpose. I intend to return to Ghana this summer (2011) and bring back some field collected mosquitoes with an eye toward initiating a pilot study.

Finally, there is another interesting research objective that has not been addressed in this work, but that could be followed using the techniques described herein. The possible involvement of the circadian clock in *An. gambiae* chemosensation has yet to be explored. Nearly all organisms express daily rhythms as a result of living in a world dominated by the 24-hour solar cycle (Yu and Hardin 2006). Mosquitoes display overt

rhythms in behaviors that include flight activity, bloodfeeding, oviposition, diapause, and pupal eclosion (Clements 1999; Wanji et al. 2003; Beach and Craig 1977; Charlwood and Jones 1979, 1980; Pandian 1980; Charlwood et al. 2002; Charlwood et al. 2003; Sumba et al. 2004). Many of the behavioral reports include data from diel field observations and therefore do not represent controlled laboratory experiments. Indeed few of the rhythms described in mosquitoes have been determined to be endogenous in their nature and by definition cannot accurately be described as circadian. Flight activity is perhaps the best exception of a behavior that is known to be under circadian control, showing persistent rhythms under constant lighting conditions and varying temperatures (Clements 1999; Jones 1972; Jones 1976; Jones and Reiter 1975; Jones and Gubbins 1977; Chiba and Tomioka 1985; Pandian 1994). One study also indicates that the circadian rhythm of flight behavior of the mosquito, *Culex pipiens*, is not under the control of a central clock in the optic lobe, but may reside in a decentralized oscillator that is sensitive to light (Kasai and Chiba 1987).

The *An. gambiae* flight rhythm is regulated by the circadian clock (Clements 1999). As expected for a nocturnal animal, *An. gambiae* flight activity has a free running period of less than 24 hours with a distinct peak of activity at the lights off transition (Clements 1999; Jones and Gubbins 1978). Interestingly, the flight pattern changes following insemination, with a loss of the subjective dusk activity peak and an increase in the overall

activity in scotophase (Jones and Gubbins 1978). This pattern of behavioral modification has been observed in at least one other nocturnal mosquito species, *An. stephensi* (Rowland 1989). Perhaps the enhanced activity following insemination facilitates the initiation of host seeking as females search for a blood meal, required for ovule development. Two studies have addressed the issue of host seeking in *An. gambiae* following a bloodmeal, with conflicting results. In the first, *An. gambiae* females continued to host seek, based on upwind flight in a wind tunnel, with a rhythmicity that mirrored unfed females (Klowden and Briegel 1994). In the second, *An. gambiae* females were refractory to host seeking for the first 72 hours following a bloodmeal (Takken et al. 2001).

A PubMed survey of the available scientific literature suggests that there is a lack of studies that have identified rhythms in mosquito physiology or gene expression, one exception being a recent paper that demonstrates gene rhythmicity in a *timeless* homolog in *Ae. aegypti*, the yellow fever vector (Gentile et al. 2006). The genomes of both *An. gambiae* (Holt et al. 2002) and *Ae. aegypti* (Nene et al. 2007) encode homologs of the *D. melanogaster* clock genes *clk, cyc, per, tim, cry, dbt, ck2, sgg, vri,* and *pdp1* (unpublished observation). The existence of behavioral circadian rhythms and clock gene homologs in mosquitoes strongly suggests that the circadian clock is well conserved among dipterans and that the mechanisms controlling clock outputs are likely to be similar. Whether *An. gambiae* utilizes a strong central clock like those

found in many nocturnal species such as cockroaches, or relies on distributed clocks like those generally found in diurnal species such as *D. melanogaster*, has yet to be determined.

It is worth noting that the blood sucking sandfly, *Lutzomyia longipalpis*, a vector for leishmaniasis, exhibits circadian locomotor activity and rhythmic expression of clock genes *per*, *tim*, and *cyc* in heads (Meireles-Filho et al. 2006a,b). The activity rhythm is depressed after blood feeding and the expression of *per* and *tim* are also downregulated (Meireles-Filho et al. 2006a). The potential for circadian/post-blood feeding regulation of olfactory sensitivity have not been studied in the sandfly. Nonetheless it can be speculated that the observed post-blood meal downregulation of clock genes may lead to sensory depression via regulation of clock-controlled olfactory genes. This is a testable hypothesis that, if supported, could form the basis for a reduction in host seeking that activity depression implies.

References

Beach RF and Craig GB (1977) Night length measurements by the circadian clock controlling diapause induction in the mosquito *Aedes atropalpus*. *J Insect Phys* 23: 865-70.

Benton R, Vannice KS, Gomez-Diaz C, and Vosshall LB (2009) Variant ionotropic glutamate receptors as chemosensory receptors in Drosophila. *Cell* 136: 149-62.

Charlwood JD et al. (2003) Observations on the swarming and mating behaviour of *Anopheles funestus* from southern Mozambique. *Malar J* 2:2.

Charlwood JD et al. (2002) The swarming and mating behaviour of *Anopheles gambiae* s.s. (Diptera: Culicidae) from São Tomé Island. *J Vector Ecol* 27:178-83.

Charlwood JD and Jones MDR (1980) Mating in the mosquito, *Anopheles gambiae* s.l. 2. Swarming behavior. *Physiol Entomol* 5: 315-20.

Charlwood JD and Jones MDR (1979) Mating-Behavior in the mosquito, *Anopheles gambiae* s.l. 1. Close range and contact behavior. *Physiol Entomol* 4: 111-20.

Chiba Y and Tomioka K (1985) *"Physiological mechanisms of entrainment of mosquito and cricket circadian activities to light-dark cycle."* Circadian Clocks and Zetgebers: Proceedings of the first Sapporo symposium on biological rhythm. Hokkaido Press.

Clements AN (1999) **The Biology of Mosquitoes. Volume 2**. Chapman and Hall.
Holt RA et al. The genome sequence of the malaria mosquito *Anopheles gambiae*. *Science* 2002, 298(5591):129-49.

Gentile C et al (2006) Cloning and daily expression of the *timeless* gene in *Aedes aegypti* (Diptera:Culicidae). *Insect Biochem Mol Biol* 36: 878-84.

Jones MDR and Gubbins SJ (1978) Changes in the circadian flight activity of the mosquito *Anopheles gambiae* in relation to insemination, feeding and oviposition. *Physiol Entomol* 3: 213-20.

Jones MDR et al. (1972) The circadian rhythm of flight activity of the mosquito *Anopheles gambiae*: the light response. *J Exp Biol* 57: 337-46.

Jones MDR (1976) Persistence in continuous light of a circadian rhythm in the mosquito *Culex pipiens fatigans* Wied. *Nature* 261: 491-2.

Jones MDR and Reiter P (1975) Entrainment of the pupation and adult activity rhythms during development in the mosquito *Anopheles gambiae*. *Nature* 254: 242-4.

Jones MDR and Gubbins SJ (1977) Modification of circadian flight activity in the mosquito *Anopheles gambiae* after insemination. *Nature* 268:732.

Kasai M and Chiba Y (1987) Effect of optic lobe ablation on circadian activity in the mosquito, *Culex pipiens pallens*. *Physiol Entomol* 12: 59-65.

Klowden MJ and Briegel H (1994) Mosquito gonotrophic cycle and multiple feeding potential: contrasts between Anopheles and Aedes (Diptera: Culicidae). *J Med Entomol* 31: 618-22.

Meireles-Filho AC et al. (2006a) The biological clock of an hematophagous insect: locomotor activity rhythms, circadian expression and downregulation after a blood meal. *FEBS Lett* 580: 2-8.

Meireles-Filho AC et al. (2006b) Rhythmic expression of the cycle gene in a hematophagous insect vector. *BMC Mol Biol* 7: 38.

Nene V et al (2007) Genome sequence of *Aedes aegypti*, a major arbovirus vector. *Science* 316: 1718-23.

Pandian RS and Chandrashekaran MK (1980) Rhythms in biting behavior of a mosquito, *Armigeres subalbatus*. *Oecologia* 47: 89-95.

Pandian RS (1994) Circadian rhythm in the biting behaviour of a mosquito *Armigeres subalbatus* (Coquillett). *Indian J Exp Biol* 32: 256-60.

Rowland M (1989) Changes in the circadian flight activity of the mosquito *Anopheles stephensi* associated with insemination, blood-feeding, oviposition and nocturnal light intensity. *Physiol Entomol* 14: 77-84.

Sumba LA et al (2004) Daily oviposition patterns of the African malaria mosquito *Anopheles gambiae* Giles (Diptera: Culicidae) on different types of aqueous substrates. *J Circ Rhyth* 2: 1-7.

Takken W et al. Inhibition of host-seeking response and olfactory responsiveness in *Anopheles gambiae* following blood feeding. *J Insect Physiol*. 2001, 47(3):303-310.

Wanji S et al. (2003) Anopheles species of the mount Cameroon region: biting habits, feeding behaviour and entomological inoculation rates. *Trop Med Int Health* 8: 643-9.

Yu W and Hardin P (2006) Circadian oscillators of Drosophila and mammals. *J Cell Sci* 119: 4793-95.