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Gustatory Receptors in Mosquito Olfaction and Host-Seeking Behavior

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The text of this dissertation, in part, is a reprint of material as it appears in the paper "Targeting a dual detector of skin and CO_2 to modify mosquito host seeking" (*Cell* 155:1365-1379). In particular, material from that publication is included throughout Chapter 2 and in Fig. 4.2. The co-authors Dyan MacWilliam, Sean Boyle, and Tom Guda listed in that publication contributed original research to the publication that is not included in this dissertation. The co-author Anandasankar Ray listed in that publication directed and supervised the research that forms the basis for this dissertation.

ABSTRACT OF THE DISSERTATION

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by

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Female mosquitoes have evolved multiple strategies to find hosts from a distance by their odor. Few compounds from the human odor blend are known to mediate these behaviors. One is carbon dioxide (CO_2), which activates resting mosquitoes and triggers upwind flight. CpA neurons on the maxillary palps express three members of the gustatory receptor (GR) family and detect CO_2.

I found that CO_2-sensitive cpA neurons in both *Aedes aegypti* and *Anopheles gambiae* also detect components of the human odor blend. CpA responses to these odorants closely resemble its responses to CO_2, and when CO_2 and skin odorants are presented together, cpA responds more strongly to the combination than to either alone. CpA also detects the natural human odor blend. A novel long-term inhibitor of cpA was used to block cpA activity, and behavioral tests in cpA–off mosquitoes revealed specific deficits in behavioral activation in response to odor, even without a CO_2 stimulus.

Genes for the receptor subunits Gr1, 2, and 3 were cloned and expressed in the *Drosophila* ab1C neuron. In mutants lacking *Drosophila* receptors, Gr2 and Gr3 form a functional receptor that is as sensitive to CO_2 and more sensitive to other odorants than when Gr1 is added. Two inhibitors reduced CO_2 responses in neurons expressing Gr2+3, and adding Gr1 modulated their activity in an odorant-specific manner. When mosquito GRs are expressed with *Drosophila* GRs, they form a CO_2 receptor only when both mosquito Gr1+2 and *Drosophila* Gr63a are present, providing clues to the evolutionary history of protein interactions in this receptor class.

Mosquitoes use odor to discriminate between more and less preferred hosts. Human participants were ranked in attractiveness in wild type and mutant mosquitoes lacking function in cpA or other olfactory neurons. Mutants had altered preferences from wild type. Additional studies may elucidate the mechanisms by which these receptors contribute to preference.

The results of this study integrate molecular, physiological, and behavioral experiments to decode more of the links between a mosquito's chemical environment and her behavioral output. Better understanding the mechanisms driving host-seeking behavior will contribute to critically needed new strategies for combating mosquito-borne disease.

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Chapter 1. Mosquito olfaction and host-seeking behavior.

Mosquitoes and the diseases they carry are a major public health threat to human populations. Malaria (transmitted by *Anopheles* mosquitoes) kills over a million people every year, mostly children in sub-Saharan Africa (Murray et al. 2012). Dengue viruses (transmitted primarily by *Aedes* mosquitoes) cause almost 100 million cases every year, mostly in the tropics, representing a major health and economic burden that falls disproportionately on developing countries (Bhatt et al. 2013). There is an urgent need to understand and manipulate mosquito physiology and behavior to control these and other mosquito-borne diseases around the world.

Female mosquitoes of most species feed on vertebrate blood for egg development. To transmit disease, a female mosquito must blood-feed twice: once to acquire the pathogen from an infected host, and again, after an incubation period during which the pathogen infects the mosquito and travels to the salivary glands, to transmit the pathogen to another host. Because of this requirement, anything that reduces the ability of mosquitoes to successfully find hosts and blood feed will reduce disease transmission by a squared factor. Likewise, improving our understanding of how mosquitoes use odors to find hosts will improve trapping strategies for vector surveillance and control.

How, then do mosquitoes find their hosts? At short range, they use a variety of cues, including humidity, temperature, and visual cues along with odors to home in on a host and select a biting site. From a distance, odors are the only cues available. To reach a host, then, mosquitoes must complete several distinct tasks in sequence, including activation (initiation of flight), anemotaxis (orientation upwind), and landing (Cardé and

Gibson 2010). Along the way, they may have to choose between multiple acceptable hosts and avoid unacceptable hosts. Current evidence indicates that all of these behaviors – activation, anemotaxis, landing, preference, and repellency – are guided by distinct sets of olfactory cues detected by independent olfactory pathways.

Model species for host-seeking behavior

Mosquitoes are a diverse family of about 3,500 described species that live in different habitats around the world and have a variety of ecological specializations, and only some of which feed on vertebrate blood (Harbach 2007). Even among those that do feed on vertebrate blood, a range of host specializations have been observed, from species that will opportunistically feed on any available vertebrate to those that prefer a specific class of vertebrate, such as mammals or birds, to those that specialize on a certain vertebrate species, human or otherwise. Mosquitoes that mostly feed on human hosts are called anthropophilic (versus ornithophilic for bird specialists or zoophilic for others). These are the most important vectors of human disease, but those that sometimes feed on other vertebrates also threaten public health, as they can transmit zoonoses to humans.

Due to their ease of being reared in the laboratory and their importance as disease vectors, three species have emerged as the premier models of mosquito host-seeking behavior. The yellowfever mosquito *Aedes* (*Stegomyia*) *aegypti* (L., 1762) is a cosmotropical, day-biting anthropophilic species that outside of its native range in Western Africa is almost exclusively urban and spends its entire life cycle in close

proximity with human habitations. This mosquito is a primary vector of yellow fever, dengue, and chikungunya viruses.

Anopheles gambiae Giles 1902 *sensu* Mattingly, 1977, is part of the sub-Saharan *An. gambiae* complex of cryptic species and includes two molecularly and ecologically distinguishable forms that are sometimes considered incipient species: *Anopheles gambiae sensu stricto* (formerly the S form) and *Anopheles coluzzii* Coetzee & Wilkerson, 2013 (formerly the M form). This species (or these species) are highly anthropophilic, breed in human-modified landscapes, and feed at night, often by entering homes. They are primary vectors of human malaria.

The southern house mosquito *Culex quinquefasciatus* Say, 1823 (formerly known as *Culex fatigans* Wiedemann, 1828 or *Culex pipiens ssp*. *quinquefasciatus*) is found throughout the tropics and sub-tropics. This species is ornithophilic in much of North America (Allan et al. 2006, Elizondo-Quiroga et al. 2006, Savage et al. 2007), but anthropophilic or zoophilic in other regions (Mboera and Takken 1999, Muturi et al. 2008). Cx. quinquefasciatus is a major vector of bancroftian filariasis in South Asia and West Nile Fever in North America.

Composition and origins of human odor

Human odor is a complex blend. Over 700 compounds have been detected by different researchers (Conkle et al. 1967, Perry et al. 1970, Ellin et al. 1974, Krotoszynski et al. 1977, Zeng et al. 1991, Bernier et al. 1999, Bernier et al. 2000, Healy and Copland 2000, Healy et al. 2002, Ghaninia et al. 2008, Verhulst et al. 2009, Verhulst 2010, reviewed in Dormont et al. 2013, see also Appendix 3). These range from small, polar

compounds like carbon dioxide and ammonia to large, barely volatile chemicals like androstenone, with many different acids, alcohols, ketones, and other compounds in between. A comparison among several independent analyses of human odor identified 25 "most frequently isolated" compounds that may form the core of human scent; these are structurally diverse and generated by a number of different biochemical pathways (Dormont et al. 2013). The exact blend of odorants changes depending on location on the body, age and sex of the person sampled, and hygiene. There is also considerable variation between individuals, including an "odor fingerprint" that dogs and other animals use to identify individual humans by scent, and an "odor barcode" in the ratios of certain characteristic odorants that in the future may allow forensic scientists to identify individuals by scent (Curran et al. 2010b, a).

The most obvious sources of human odor are sweat and breath, which, among other functions, expel byproducts of our own metabolism—like carbon dioxide, which is generated in all our cells and voided in breath, or lactic acid, a major component of eccrine sweat. Other compounds, including androstenone, are secreted by apocrine sweat glands and are proposed to function as human pheromones. In addition to eccrine and apocrine sweat glands, sebaceous glands secrete waxy sebum and have a distinct distribution across the body.

Recently, the Human Microbiome Project has shown that different regions of the body provide distinct microclimates and are home to divergent populations of microbes (reviewed in Grice and Segre 2011). Their biochemical diversity contributes many, if not most, of the distinctive compounds that make up human odor. Microbial action has been

strongly implicated in generating odorants attractive to mosquitoes: *Anopheles gambiae* mosquitoes were more attracted to incubated sweat samples than fresh samples in a dual-port olfactometer (Meijerink et al. 2000), and are likewise attracted to headspace of microbes cultured from feet (Verhulst et al. 2009, 2010, Verhulst 2010).

Organization of the mosquito olfactory system

Mosquitoes use their antennae, maxillary palps, and labella to detect odors. Each of these appendages is decorated with olfactory sensilla, specialized hairs with cuticular pores that allow odorants to enter. The cell bodies of 2–5 olfactory receptor neurons (ORNs) are located beneath the sensillum and project branched dendrites into the interior of the sensillum, an electrically isolated region where they are bathed in sensillar lymph (McIver 1982, Kaissling 1985). Pores in the cuticle of the sensillum allow airborne odorants access to the inside of the sensillum. Odorant-binding proteins (OBPs) in the lymph likely facilitate the movement of mostly non-water-soluble odorants to receptors in ORN plasma membranes; whether these proteins also play a role in odor coding is debated.

Much of what is known about ORN function was first described in *Drosophila melanogaster*, but many of the same organizing principles also apply in mosquitoes. In both insects, most ORNs have spontaneous activity, i.e., they fire action potentials at low levels in the absence of any olfactory stimuli. A single ORN can be activated or inhibited by a number of different odorants, and a single odorant can activate or inhibit multiple ORN classes simultaneously depending on concentration, resulting in a systems-level combinatorial code of odor quality and intensity (Hallem and Carlson 2006, Carey et al.

2010, Wang et al. 2010). ORNs fall into distinct functional classes defined by the set of receptors they express, and ORNs of different classes are co-located in sensilla in stereotyped combinations (de Bruyne et al. 2001, Couto et al. 2005). The axons from all the ORNs expressing the same receptors converge in a distinct anatomical region of the brain antennal lobe called a glomerulus (Couto et al. 2005, Fishilevich and Vosshall 2005, Ignell et al. 2005, Ghaninia et al. 2007a, Ghaninia et al. 2007b).

Mosquitoes use receptors from at least three chemoreceptor families in olfaction: the odorant receptors (ORs), the gustatory receptors (GRs), and the ionotropic receptors (IRs). Odorant receptors always come as heteromers of unknown stoichiometry consisting of an obligatory co-receptor, orco (previously designated Or7), and an additional OrX receptor that confers odorant specificity. Expressing orco + OrX combinations in cell culture confers odorant responses; this method has been used to decode a substantial fraction of the OR repertoire of *An. gambiae* mosquitoes (Wang et al. 2010). OrX genes can also be expressed in an "empty neuron" in *Drosophila melanogaster* that is missing its endogenous receptor. There they co-locate with the *Drosophila* orco and confer receptor-specific properties to the neuron such as spontaneous firing frequency, odorant specificity, and dose responses (Hallem et al. 2004). This system has been used for large-scale analysis of *An. gambiae* OR tuning properties, which has revealed a number of receptors that respond to compounds found in human odor or have otherwise been implicated in mosquito behavior (Carey et al. 2010).

GRs are a chemoreceptor family related to the ORs and are primarily involved in taste sensation. Three GRs are involved in olfaction: they are designated Gr22, Gr23,

and Gr24 in anophelines and Gr1, Gr2, and Gr3 in other mosquitoes. These are all expressed in one of the three neuronal classes found in the capitate peg sensilla on the maxillary palp, the cpA neuron, which detects CO_2 and other host odors (Jones et al. 2007, Lu et al. 2007, Syed and Leal 2007, Robertson and Kent 2009, Tauxe et al. 2013).

Ionotropic receptors are an evolutionarily distinct lineage of chemoreceptors in protostomes that evolved from ionotropic glutamate receptors. Multiple IRs seem to be required for olfactory function, and a few have been described as facultative co-receptors. Both larval and adult mosquito antennae express IRs. They have not yet been fully characterized, but many IRs in *D. melanogaster* and mosquitoes detect polar compounds such as acids, ammonia, and amines, and it is likely that mosquito IRs serve similar functions (Croset et al. 2010, Liu et al. 2010).

The several hundred olfactory sensilla of the antenna come in a variety of morphological and functional classes and are presumed to express various combinations of ORs and IRs. The maxillary palps have 8–102 sensilla, depending on sex and species, of a single class designated capitate peg (cp) because of its shape (McIver 1982). Each cp sensillum houses dendrites of three neurons; the largest of these, cpA, is the GR-expressing CO_2-sensitive neuron mentioned above. One of the other cp neurons (cpB in *An. gambiae* and *Cx. quinquefasciatus* and cpC in *Ae. aegypti*) expresses the relatively conserved Or8 along with orco and detects R-(−)-1-octen-3-ol (Lu et al. 2007, Syed and Leal 2007). Olfactory responses and receptors have also been detected in the labellum, but these are not well characterized (Kwon et al. 2006, Bohbot et al. 2007).

A minimal stimulus induces host-seeking behavior

Three cues, in combination, are sufficient to induce females of *Ae. aegypti* and *Aedes albopictus* (Skuse, 1894) to perform the entire suite of host-seeking behaviors, from activation to landing and probing at a surface (Klun et al. 2013): CO_2 induces activation and upwind flight, and the combination of heat and water vapor induces landing and probing. If any one of these is missing, even in an artificial environment with no other host-related stimuli, mosquitoes become dramatically less successful at finding a simulated host. This result does not mean that CO_2, water, and heat are the only cues mosquitoes use to find hosts—this is easily refuted by observing robust mosquito attraction to human skin residues that give off very little or no CO_2, water, or heat. It does mean that mosquitoes use different cues at different distances from the host such that no one cue is sufficient to lure a mosquito. Indeed, McMeniman et al. (2014) demonstrated that even at short range, at least two out of the three stimuli of heat, skin odor, and CO_2 must be present to induce blood feeding in *Ae. aegypti*. Because of this, it is virtually meaningless to discuss whether any single cue is "attractive." Instead, the cues that mediate each stage of the behavioral program and the sensory pathways that detect them will be discussed.

Activation

A female mosquito at rest tends to remain at rest, but she can be activated either by odors or by stochastic events whose frequency depends on time of day. Female *Ae. aegypti* have a low basal rate of spontaneous locomotion that varies in a circadian rhythm, peaking in late afternoon (Taylor and Jones 1969). This spontaneous rhythm is

modified by physiological and developmental status (Jones 1981) and by infection with *Wolbachia* or dengue (Evans et al. 2009, Lima-Camara et al. 2011). In the presence of CO_2 or exhaled breath, mosquitoes take off at a dramatically higher rate and, if there is a breeze, immediately fly upwind (Gillies 1980). Even very small changes in CO_2 concentration are sufficient to induce this behavior: for example, Eiras and Jepson observed activation in response to concentrations of CO_2 a mere 0.03% above background levels in *Ae. aegypti* (1991) and Healy and Copland observed activation with 0.01% above background in *An. gambiae* (Healy and Copland 1995). Human breath contains about 4% CO_2, and if this is scrubbed out, the remaining odors do not induce activation in *Ae. aegypti* (Klun et al. 2013). However, *Ae. aegypti* does activate when exposed to a natural blend of human skin odor without added CO_2, although to a lesser extent than to breath or CO_2 (Tauxe et al. 2013).

CO_2 is detected by a single class of ORNs designated cpA. These are the large GR-expressing neurons in the capitate peg sensilla on the maxillary palp. Exclusively in blood-feeding species, this neuron has an unusual lamellate dendrite that presumably serves to increase membrane surface area and therefore odor sensitivity (McIver 1972, 1982, Lu et al. 2007). In *Ae. aegypti*, cpA neurons are silent in CO_2-free air and begin to fire when CO_2 concentrations reach 150–600 ppm (Grant et al. 1995). CpA encodes both constant CO_2 concentrations and fluctuations in CO_2 concentration as low as 50 ppm with a phasic–tonic firing pattern (Grant et al. 1995). In addition to CO_2, cpA also detects human skin odor. CpA detects several components of the human odor blend are

implicated in both *Ae. aegypti* and *An. gambiae*. It responds to these and related odorants with a phasic–tonic response very similar to its CO_2 responses (Tauxe et al. 2013).

The receptors expressed in this neuron are designated Gr1, Gr2, and Gr3 in culicine mosquitoes (Syed and Leal 2007, Robertson and Kent 2009) and Gr22, Gr23, and Gr24 in anophelines (Jones et al. 2007, Lu et al. 2007). Unlike most ORs, these receptors are highly conserved and orthologs can be found not just in mosquitoes but across many orders of holometabolous insects (Robertson and Kent 2009). Gr3 is required for cpA responses to CO_2 (McMeniman et al. 2014) as well as other odorants (Tauxe, unpublished data). The relative contributions of Gr1 and Gr2 to odor detection are still being decoded (see Chapter 3).

CpA activity is tightly linked with behavioral activation. It has long been known that CO_2 is sufficient to trigger activation and subsequent upwind flight (Gillies 1980). Recent findings show that cpA activity is also necessary for odor-mediated activation: when the neuron is silenced by either chemical or genetic means, activation rates in the presence of CO_2 or skin odors go down to the basal rate observed in the absence of odors (Tauxe et al. 2013, McMeniman et al. 2014).

In the absence of odor, the probability of a mosquito taking off is constant over time scales of an hour or so, suggesting that this is triggered by stochastic events at a single command center (i.e., not due to odor stimuli) (Daykin et al. 1965). The putative command center appears to be independent of the CO_2 pathway, since diel variation in spontaneous locomotor activity is not affected in Gr3 mutant mosquitoes (McMeniman et al. 2014). Likewise, mosquitoes that take off in the absence of odor do not immediately

turn upwind, but engage in undirected flight CpA-silenced mosquitoes that activate stochastically, however, do find and land on host odor sources, indicating that cpA is not required for host-seeking behaviors that occur after activation (Tauxe et al. 2013, McMeniman et al. 2014).

Anemotaxis

Mosquitoes use a combination of olfactory and visual cues to navigate upwind toward a host. In the presence of odor cues, female mosquitoes use visual cues as landmarks to guide upwind flight: they adjust their flight to maintain a steady front-to-back visual flow of landmarks either above or below them, resulting in upwind flight with a steady ground speed. This strategy, known as optomotor anemotaxis, was first described in *Ae. aegypti* (Kennedy 1939).

When a flying *Ae. aegypti* contacts a filament of CO_2 and thus experiences an increase in CO_2 concentration, she immediately turns upwind and increases speed for part of a second or until contacting another filament of CO_2 . If she does not soon contact a CO_2 filament, she ceases upwind flight and casts across the direction of airflow in a manner that maximizes the chance of re-encountering a filament from the same source (Dekker and Cardé 2011). This surge–cast strategy is highly effective at bringing the mosquito close to a source of turbulent CO_2 and closely resembles the strategy male moths use to find a pheromone-emitting female (Cardé and Willis 2008). Upwind surging resembles the activation of a resting mosquito by CO_2 or skin odor, in which a mosquito takes off and immediately flies directly upwind, and it also depends on cpA:

chemically treated mosquitoes whose cpA neurons do not respond to changing CO_2 concentrations cannot track a CO_2 plume (Turner et al. 2011).

Because the CO_2-sensitive cpA neuron does not discriminate between whole skin odor and a low concentration of CO_2, it is expected that a skin odor plume would also trigger upwind surging behavior. Indeed, when a female *Ae. aegypti* enters a plume of undiluted skin odor, she turns upwind and increases in flight speed just as she does in a plume of CO_2 (Dekker et al. 2005, Dekker and Cardé 2011).

That said, cpA activation by skin odor does not explain other aspects of upwind flight toward a skin odor source. First, although diluted skin odor is not sufficient to activate a mosquito or induce an upwind surge, it is highly attractive after a mosquito is activated by a momentary pulse of CO_2 (Dekker et al. 2005). Second, when separate CO_2 and skin odor plumes are presented to a mosquito simultaneously, she will ignore the CO_2 plume and navigate toward the human odor source regardless of how the odor plumes are arranged (Lacey et al. 2014). Finally, mosquitoes lacking cpA function that activate stochastically navigate efficiently to a source of skin odor (Tauxe et al. 2013) and show only mild impairment at finding a human in a large semi-field enclosure (McMeniman et al. 2014). All of these observations indicate that an additional, unidentified olfactory pathway involved in upwind flight toward skin odor.

The details of how mosquitoes adjust their flight patterns in response to host odors do vary by species. In *Cx. quinquefasciatus*, CO_2 activates mosquitoes just as in *Ae. aegypti*, but it does not induce an upwind surge; rather, these mosquitoes tend to fly upwind regardless of what odors are present. Adding a human odor source actually slows

upwind flight and induces landing, but only after mosquitoes have flown past it and doubled back (Lacey and Cardé 2011). This difference may be because *Cx. quinquefasciatus* has a broader host range and is not as attracted to human odor as *Ae. aegypti*, or it may be an adaptation to avoid host defensive behaviors. Regardless, the differences in behavior toward human skin odor or CO_2 again argues that an unidentified, non-cpA olfactory pathway must be involved.

In *An. gambiae*, Spitzen et al. (2013) found that in the absence of host-related odors, mosquitoes in a dark wind tunnel will fly directly upwind, whereas mosquitoes that had contacted a plume of human odor turned frequently so that they flew mostly crosswind while moving slowly upwind until they came close to the end of the arena and/or a heat source . The authors suggest that this may be an adaptation for this nocturnal species to be able to find hosts in the dark by intensively scanning the environment. It would be helpful to conduct similar experiments with CO_2 plumes and with low light to investigate the relative contributions of CO_2 and optomotor anemotaxis in host finding in this species.

Landing

In *Ae. aegypti*, heat and moisture together are sufficient to induce landing behavior and subsequent probing, but neither stimulus by itself is sufficient (Klun et al. 2013). Human odor in the absence of these cues is also sufficient to induce landing, and landing is enhanced with added heat (Schreck et al. 1990). Mosquitoes can also discriminate odor collected from different parts of the human body, which may contribute

to landing site selection (Schreck et al. 1990). The components of human odor that induce this landing behavior in *Ae. aegypti* have not been identified.

Sweat also enhances the attractiveness of a warm, moist cue in *An. gambiae*, and this has been attributed at least partly to the presence of C4–C6 2-oxocarboxylic acids (Healy and Copland 2000, Healy et al. 2002).

The receptors for heat and moisture are not known in mosquitoes, although in other insects they belong to the transient receptor potential (TRP) and pickpocket (ppk) families (Liu et al. 2007, Cameron et al. 2010, Fowler and Montell 2013).

Trap Catch

Presumably, an odor blend that includes or mimics the most important cues from an attractive host would trap as many mosquitoes as a live host. Conversely, compounds that increase trap catch can provide clues for what odors attract different kinds of mosquitoes. Unfortunately, most trapping studies inherently report only a single value: the number of mosquitoes caught by a particular type or arrangement of traps. While this is certainly the most relevant number for many purposes, it obscures the mechanistic details of how odors interact to attract mosquitoes. "Attractive" odors could lure mosquitoes from a greater distance, arrest mosquitoes at the trap entrance where they can be sucked into the trap, induce mosquitoes to enter the trap themselves, or simply increase the salience of other cues, and all of these effects would result in a higher overall catch.

In side-to-side comparisons, the most effective mosquito lures yet devised attract fewer anthropophilic mosquitoes than a living person (Okumu et al. 2010) (reviewed in

Mukabana et al. 2010). CO_2 is routinely used in mosquito traps for vector surveillance and control. This general host cue attracts many hematophagous insects, including many mosquitoes, but it is much less effective at attracting anthropophilic mosquitoes than a human bait (Costantini et al. 1996) and not very effective at all for trapping medically important *Aedes* species like *Ae. aegypti* and *Ae. albopictus* (personal communication, M. E. Metzger, California Department of Health). Because of the logistical difficulties of using CO_2 from pressurized cylinders or from dry ice or in rural areas, especially in developing countries, alternative strategies have been proposed including fermenting sugar or molasses with yeast that generate CO_2 (Smallegange et al. 2010b, Mweresa et al. 2014) or by using other chemicals that activate the same mosquito ORN as CO_2 (Tauxe et al. 2013). When used by itself, CO_2 presumably induces similar behaviors to those seen in laboratory assays: it activates host-seeking mosquitoes and induces them to fly upwind toward the trap. It is not sufficient, however, to induce a mosquito to enter a trap: when *Culex* mosquitoes were video-taped interacting with CO_2 traps, it was seen that they would reach the vicinity of the trap and stay there flying around the entrance until only some of them were sucked into the trap by the fan (Cooperband and Cardé 2006).

Humans secrete an unusual amount of lactic acid in our skin compared to other large vertebrates, predominantly the L-(+) enantiomer, so this is considered a human-specific cue of potential use to anthropophilic mosquitoes (Acree et al. 1968, Smith et al. 1970, Dekker et al. 2002). Early laboratory studies found that L-(+)-lactic acid attracted large proportions of *Ae. aegypti* mosquitoes, but only when combined with CO_2 (Acree et al. 1968, Smith et al. 1970). This was measured by entry into a trap port on the side of

the olfactometer, so CO_2 by itself was not found to be attractive. Enzymatically reducing the lactic acid content of skin odor extracts reduced the attractive response, and the L-(+) enantiomer was about five times more attractive than the D-(−) isomer (Acree et al. 1968). Lactic acid has also been implicated in mosquito preference for humans over other animals and for preference for some humans over other humans (discussed in the Preference section below).

Lactic acid is detected by ORNs in grooved peg sensilla on the antennae. A subset of the ORNs in these sensillae are activated by both L-(+) and D-(−) enantiomers of lactic acid; a different subset are inhibited by both (Davis and Sokolove 1976). The basis for mosquitoes' behavioral discrimination between enantiomers is unknown. Sensitivity to lactic acid in the grooved peg sensillae varies during the adulthood of a mosquito and seems to correlate with variation in behavioral responsiveness to host odor during maturation and through the gonotrophic cycle (Davis 1984). The receptor or receptors expressed in these ORNs have not yet been identified, despite extensive screening of ORs (Carey et al. 2010, Wang et al. 2010). Mosquito grooved peg sensilla appear to be homologous to fly coeloconic sensilla, which express IR-family chemoreceptors and respond to acids (Benton et al. 2009), so it is likely that the mosquito lactic acid receptor is also a member of this family.

Ammonia may also attract some mosquitoes. Braks et al. (2001) observed that incubated sweat was more attractive to *An. gambiae* mosquitoes than fresh sweat in a two-port olfactometer, which they attributed to strongly increased concentrations of ammonia, probably due to microbial breakdown of urea. Indeed, ammonia attracted

mosquitoes in a dose-dependent manner when presented in either aqueous or gaseous forms. Working with *Ae. aegypti* in a Y-tube olfactometer with controlled airflow to simulate upwind flight conditions, Geier et al. (1999b) observed that ammonia was not attractive by itself (in contrast to the results with *An. gambiae*) and did not increase attractiveness of CO_2, but did increase the attractiveness of lactic acid in the absence of a CO_2 plume.

Ammonia-sensitive ORNs have been observed in grooved-peg sensilla (Davis and Bowen 1994, Qiu et al. 2006) and trichoid sensilla (Qiu et al. 2006), although the high concentration of ammonia that Qiu et al. (2006) used to survey these sensilla means that the observed responses are probably not specific. It was recently shown in *Drosophila* that the ammonium transporter Amt is required for the ac1 neuron to detect ammonia (Menuz et al. 2014); this gene is also expressed in the mosquito antenna and confers ammonium responses in cell culture, which would correspond with a role in mosquito olfaction as well (Pitts et al. 2014). It is not known which mosquito ORNs or receptors determine behavior toward ammonia.

Limburger cheese has a strong odor reminiscent of "toe jam." *An. gambiae* mosquitoes, which are attracted to human feet more than to other body parts (de Jong and Knols 1996, Dekker et al. 1998) are also attracted to Limburger cheese in a dual-port olfactometer (Knols and de Jong 1996). One of the bacteria used in the production of Limburger, *Brevibacterium linens*, is an Actinomycetales closely related to the foot bacterium *Brevibacterium epidermidis*. Both of these species produce methanethiol and carboxylic acids that generate the characteristic odor (Knols and de Jong 1996).

Chemical fractionation and analysis of cheese headspace odor found a number of short-chain aliphatic acids that contribute to mosquito attraction. A synthetic blend of 12 of these acids was able to recapitulate the level of attraction observed with whole cheese (Knols et al. 1997).

In fact, many components of human odor are generated by skin-associated bacteria, and bacterial cultures from human skin attract mosquitoes (Verhulst et al. 2009). Some compounds isolated from skin-associated bacteria, particularly 3-methyl-1-butanol are believed to improve trap catch of *An. gambiae* and are used in lures blended with CO_2, ammonia, lactic acid, and tetradecanoic acid (Verhulst et al. 2011, Mweresa et al. 2014).

A variety of plant and animal sources, including humans, give off 1-octen-3-ol, primarily the (*R*)-(–) enantiomer (Hall et al. 1984). Some species of tsetse are strongly attracted to both enantiomers of this compound, which is found in high concentrations in the breath of ruminants such as cattle (Torr and Solano 2010). Octenol has also been used in trapping mosquitoes. It is not effective as a bait when used by itself, but increases trap catch of some mosquitoes when combined with CO_2 (Takken and Kline 1989). Different mosquito species respond to the combination of octenol + CO_2 quite differently: some species, primarily *Aedes*, are caught in higher numbers in traps baited with the combination than with CO_2 alone, some are caught in equal numbers, and a few, primarily *Culex* species, seem to be repelled by octenol (Takken and Kline 1989, Kemme et al. 1993, Kline 1994, Becker et al. 1995, Burkett et al. 2001).

Octenol is detected by the Or8-expressing neuron in capitate peg sensilla of the maxillary palp, the same sensillum that also houses the CO2-sensitive ORN. This ORN is designated cpB in *An. gambiae* and *Cx. quinquefasciatus* and cpC in *Ae. aegypti* (Lu et al. 2007, Syed and Leal 2007, Bohbot and Dickens 2009). It is more than two orders of magnitude more sensitive to the (*R*)-(–) enantiomer than to the (*L*)-(+) enantiomer and increases in sensitivity as an adult mosquito matures and begins host seeking (Bohbot and Dickens 2009, Bohbot et al. 2013). At least 10 other mosquito ORs also detect 1-octen-3-ol and may also influence behavior (Carey et al. 2010).

Preference

Why mosquitoes prefer to bite some people over others is one of life's persistent questions. Virtually everyone has a pet hypothesis to explain this well-known phenomenon, but there are surprisingly few studies that address the question experimentally. A few possibilities can be ruled out logically. For example, it is not likely that CO_2 is a deciding factor: the concentration of CO_2 in exhaled breath is two orders of magnitude higher than the threshold for mosquito behavior, so slight variations in that concentration are undoubtedly irrelevant. Likewise, taste of blood is not a helpful discriminant, since by the time a mosquito can taste a human's blood, she has already bitten. It is possible that she may change how long she attempts to feed or how much she engorges depending on taste cues, but her saliva will already be present in the wound, causing itchiness and potentially transmitting disease. Most variation in attractiveness to mosquitoes is probably due rather to olfactory cues, and indeed skin and sweat odors isolated from different individuals consistently attract different numbers of mosquitoes in

laboratory assays with *Ae. aegypti* (Rahm 1957, Steib et al. 2001, Chapter 4) and *An. gambiae* (Dekker et al. 2002).

Host preference has been measured at many scales, from either side of a single cage of mosquitoes (e.g., Chapter 4) to different ends of a Y-tube olfactometer (e.g., Steib et al. 2001) to mosquitoes attracted to people in different tents (Lindsay et al. 2000), and while some intriguing correlations have been identified between certain host properties or odors and mosquito preference, there are relatively few mechanistic studies in this area. It is likely that multiple olfactory pathways help to determine preference between potential hosts of different species or between potential hosts of a single preferred species. Preference for one potential host species over another varies dramatically among mosquito species (reviewed in Takken and Verhulst 2013), so it is also likely that olfactory pathways involved in host preference evolve relatively quickly.

Some factors have been found that correlate with attractiveness to malaria mosquitoes, notably pregnancy, (Lindsay et al. 2000, Ansell et al. 2002) and age, but not sex (Carnevale et al. 1978). A controlled experimental study found that attractiveness of human odor to *An. gambiae* increased after subjects drank a beer-like alcoholic beverage (Lefèvre et al. 2010), but the olfactory cue(s) involved were not identified.

In addition to its use as a general attractant for trapping, L-(+)-lactic acid has been implicated in host preference in *Ae. aegypti*. In a Y-tube olfactometer, this odor is not sufficient to activate or attract *Ae. aegypti*, but synergizes with other host odors with or without added CO_2 (Geier et al. 1996). These anthropophilic mosquitoes do not respond strongly to odors from cows, goats, or cats in a Y-tube olfactometer, but are strongly

attracted to human odor. Adding lactic acid to the odors of these other mammals induces attraction similar to what is observed with human odor (Geier and Boeckh 1999, Steib et al. 2001). In the same way, mosquitoes were attracted to odor from several humans (collected as hand rubbings on glass), but they consistently preferred odor from some over others so that individual humans could be ranked in attractiveness. While again lactic acid did not attract mosquitoes by itself, adding lactic acid to odor collected from an "unattractive" individual reversed the direction of preference, so that that person became more attractive than the "attractive" person (Steib et al. 2001). Similar results have also been shown in A*n. gambiae* (Dekker et al. 2002).

An unbiased screen found 33 candidate repellents that are found in higher abundance in the odor of people whose hand odor attracted fewer *Ae. aegypti* (Logan et al. 2008). Five of those with the strongest antennal responses were used for follow-up behavioral tests: 6-methyl-5-hepten-2-one, octanal, nonanal, decanal, and geranylacetone. Only decanal was confirmed as a possible repellent.

Two more of those compounds, nonanal and 6-methyl-hepten-2-one, have been implicated by other groups as being attractive and used by mosquitoes to find preferred host species. *Cx. quinquefasciatus*, which feeds on both birds and humans, also detects aldehydes from human odor. Syed and Leal found that one ORN in the A2 trichoid antennal sensillum of this mosquito sensitively detects nonanal. Since nonanal is also found in pigeon and chicken odor, they proposed that this mosquito uses nonanal to identify potential hosts (2009).

The only olfactory molecule with a demonstrated causal role in determining host preference is orco: mutant mosquitoes lacking this co-receptor no longer prefer odor from a human over odor from a guinea pig (DeGennaro et al. 2013). Expression and allelic sensitivity of the receptor AaOr4, which requires orco to function, correlates very well with host preference for humans over guinea pigs across two subspecies of *Ae. aegypti* that differ in host preference and across hybrids of the two subspecies (McBride et al. 2014). Or4 detects 6-methyl-5-hepten-2-one (also known as sulcatone), and this compound is found at higher concentrations in the odor of humans than in other animals, so Or4 may be a key pathway for determining host preference in *Ae. aegypti*. Since mosquitoes lacking *orco* successfully discriminate among more or less attractive humans (Chapter 4), there must be an additional, non-OR pathway involved in this more challenging task.

Current progress and challenges

Mosquito host-seeking behavior has been a major subject of study for almost a hundred years, but there have been very few detailed, mechanistic studies until recently. Studies using traditional methods have relied on traps or olfactometers, which provide limited information, or correlative observations about what compound are more abundant in attractive odors and what receptors are sensitive to those odors. The advent of genetic and pharmacological tools to selectively manipulate individual neurons or receptor pathways is making it easier than ever to demonstrate causal relationships between odor and behavior. It is clear that different neural pathways are involved and redundant at

different stages of the host-seeking process (Fig. 1.1), so knocking out a single pathway is not sufficient to prevent mosquitoes from finding their hosts.

Progress is being made: it is clear that activation occurs via only two mechanisms: activation of the Gr1,2,3–expressing cpA neuron or by stochastic (non–odor mediated) activation controlled by circadian rhythms (Tauxe et al. 2013, McMeniman et al. 2014). Optomotor anemotaxis can be mediated by this pathway or another pathway that remains unidentified. Preference between hosts of different species is partially mediated by orco in *Ae. aegypti*, perhaps in conjunction with Or4, but some preference remains even when this pathway is disabled (DeGennaro et al. 2013, McBride et al. 2014). Preference between hosts of a preferred species (i.e., humans) does not depend on orco or Gr3 in *Ae. aegypti* (Chapter 4), once again implicating another pathway. A minimal stimulus for landing behavior requires either temperature and moisture together or an unidentified subset of the human odor blend. Again, orco and Gr3 are not required, implicating another pathway. The ionotropic receptor (IR) family

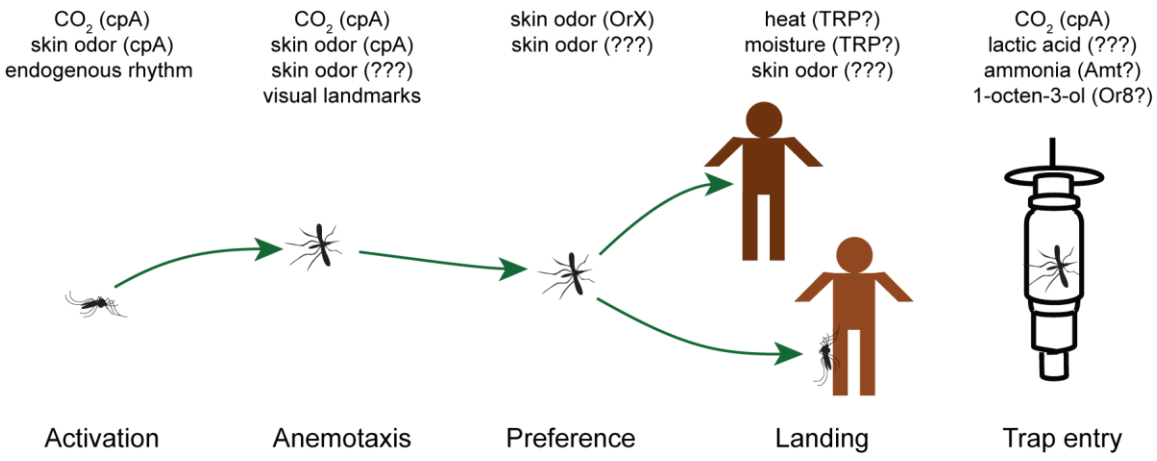


Figure 1.1. Major host cues used by mosquitoes for host-seeking behavior (and the sensory receptors and/or neurons that mediate behavior)

and the transient receptor potential (TRP) family have largely been unexplored in this system and are ripe for investigation.

Ultimately, the goal of understanding mosquito host-seeking behavior is to enhance technologies for manipulating that behavior. Effective traps are necessary for mosquito surveillance, but these are currently not available for some vector species and not available for any species in developing and rural regions most affected by vector-borne disease. Masking agents that would prevent mosquitoes from targeting humans and traps that are more attractive than humans and thus could be used to prevent mosquito bites both remain elusive, but the development of either would be a major advance for public health.

Chapter 2. A single olfactory neuron class mediates mosquito activation by both carbon dioxide and skin odor

Female mosquitoes use exhaled carbon dioxide (CO_2) and human skin odorants to select and navigate toward hosts (Gillies 1980, Mboera et al. 2000, Dekker et al. 2005, Cardé and Gibson 2010, Dekker and Cardé 2011). Of these cues, the role of CO_2 is the best understood. As far back as 1922, Rudolfs observed that in a small enclosure, CO_2 "activated the insects and caused them to display 'pleasure'" (Rudolfs 1922). The concept of behavioral "activation" by CO_2 is peculiar, but it has had staying power (reviewed in Gillies 1980). Left undisturbed, "inactive" mosquitoes are just that: they do not move, or rather, they move rarely. Their movements (generally, taking flight) are random events that follow first-order kinetics (Daykin et al. 1965). However, with the addition of a CO_2 stimulus, a mosquito becomes responsive to a variety of other host-associated cues, including heat, moisture, and odorants such as L-lactic acid (Acree et al. 1968, Smith et al. 1970, Gillies 1980, Klun et al. 2013). A recently created mutant mosquito that lacks the ability to detect CO_2 also has major deficits in behavioral responses to heat and host odor, again suggesting that the suite of host-seeking behaviors is gated by CO_2 (McMeniman et al. 2014). In addition to activating behavior toward other host cues, CO_2 also attracts mosquitoes by itself (Cardé and Gibson 2010, Klun et al. 2013).

Mosquitoes detect CO_2 using a class of olfactory receptor neurons (ORNs) designated cpA. CpA neurons are housed in capitate peg (cp) sensilla on the maxillary palps, along with two other classes of ORNs which respond to other odorants (Fig. 2.1).

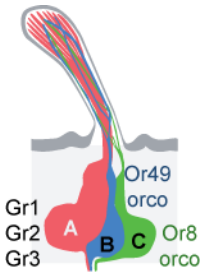


Figure 2.1. Schematic of the capitate peg sensillum in *Aedes aegypti*.

CpA neurons detect changes in CO_2 concentration as small as 50 ppm, and their phasic–tonic responses encode information both about the magnitude of changes in concentration (during the phasic response) and about the absolute concentration of CO_2 present (during the tonic response) (Grant et al. 1995, Grant and O'Connell 1996). The morphology of these ORNs is unusual, having a thick, voluminous dendrite with numerous densely packed lamellate processes at the distal end, resulting in a large membrane surface area (McIver 1972, Lu et al. 2007). This morphology is commonly associated with CO_2-sensitive neurons across blood-feeding Diptera, but is not conserved in non-blood feeders (McIver 1987). These neurons express three members of the *Gustatory receptor* (*Gr*) gene family (designated *Gr1*, *Gr2*, and *Gr3* in most mosquitoes, or *Gr22*, *Gr23*, and *Gr24* in *Anopheles*), which are conserved across many holometabolous insects and homologous to the well-known *Drosophila* CO_2 receptor made up of *Gr21a* and *Gr63a* (Jones et al. 2007, Lu et al. 2007, Syed and Leal 2007, Robertson and Kent 2009). A host-seeking female will fly upwind when these neurons are activated, toward a CO_2 source in a laboratory arena or to CO_2-baited traps in the field (Healy and Copland 1995, Dekker et al. 2005, Cooperband and Cardé 2006, Xue et al. 2008, Lacey and Cardé 2011). Conversely, preventing cpA from detecting changes in CO_2 dramatically reduces attraction toward CO_2 sources (Turner et al. 2011, Erdelyan et al. 2012).

The role of human odor in host seeking is more complex because it is a blend of hundreds of volatiles from skin, sweat, and associated microbiota (Bernier et al. 2000,

Gallagher et al. 2008, Dormont et al. 2013, see Appendix for additional references). ORNs in the antennae and palps express members of the *odorant receptor* (*Or*) and *ionotropic receptor* (*IR*) chemoreceptor families (Kwon et al. 2006, Qiu et al. 2006, Lu et al. 2007, Syed and Leal 2007, Pitts et al. 2011). Several mosquito ORs respond to skin-associated odorants and are candidates for contributing to attraction to skin (Carey et al. 2010, Wang et al. 2010). Likewise, several antennal and maxillary palp ORNs are known to respond to odorants from skin (Qiu et al. 2006, Syed and Leal 2007, Ghaninia et al. 2008).

However, a causal relationship between activity of particular receptors or neuron classes and behavioral attraction has not been established as with the cpA neuron and CO_2. Even in the presence of otherwise "attractive" stimuli such as L-lactic acid, ammonia, carboxylic acids, 1-octen-3-ol, and nonanal, a mosquito will not respond unless a CO_2 stimulus is also present (Njiru et al. 2006, Qiu et al. 2007, Syed and Leal 2009, reviewed in Smallegange and Takken 2010). The only exceptions to this rule are a small number of solvents that evoke similar behaviors as CO_2, including increasing responses to L-lactic acid (Bernier et al. 2003). Mosquitoes are nonetheless activated by and attracted to whole skin odor even in the absence of CO_2 (Schreck et al. 1981, Geier et al. 1999a, Njiru et al. 2006, Smallegange et al. 2010a, Dekker and Cardé 2011, Lacey and Cardé 2011). This suggests that some element of skin odor may also "activate" a mosquito.

The CO_2-sensitive cpA neuron also detects human odorants

The cpA neuron has long been considered a highly specialized detector of CO_2. However, recent studies have identified a small number of additional ligands that can strongly activate or inhibit this neuron or its *Drosophila* homologue (Lu et al. 2007, Turner and Ray 2009, Turner et al. 2011). Upon joining the Ray Laboratory, I identified a number of additional activators of the cpA neuron in *Aedes aegypti* (L., 1762) by screening compounds with structures similar to previously identified ligands (see Appendix 1). Surprisingly, many of these were known to be present in human odor or were structurally very similar to human odorants. This led me to hypothesize that cpA may respond generally to human-associated odorants.

A panel of odorants was selected based on reported detection in human odor or effluent, structural similarity to known ligands, and commercial availability (see Appendix 2 for details). Many of these odorants activated cpA robustly when presented at a standard dilution (10^{-2} in paraffin oil) during single-sensillum recordings (Fig. 2.2). Although *Ae. aegypti* and *Anopheles gambiae* Giles belong to divergent mosquito subfamilies, their CO_2 receptor genes are highly conserved (Robertson and Kent 2009). Accordingly, cpA responses to this panel of odorants were similar between these two species (Fig. 2.2A), suggesting a conserved role in detecting host odor. It should be noted that the odorants used in this screen vary widely in vapor pressure, so the relative sensitivities reported here do not reflect cpA's sensitivity on a molecule-per-molecule basis.

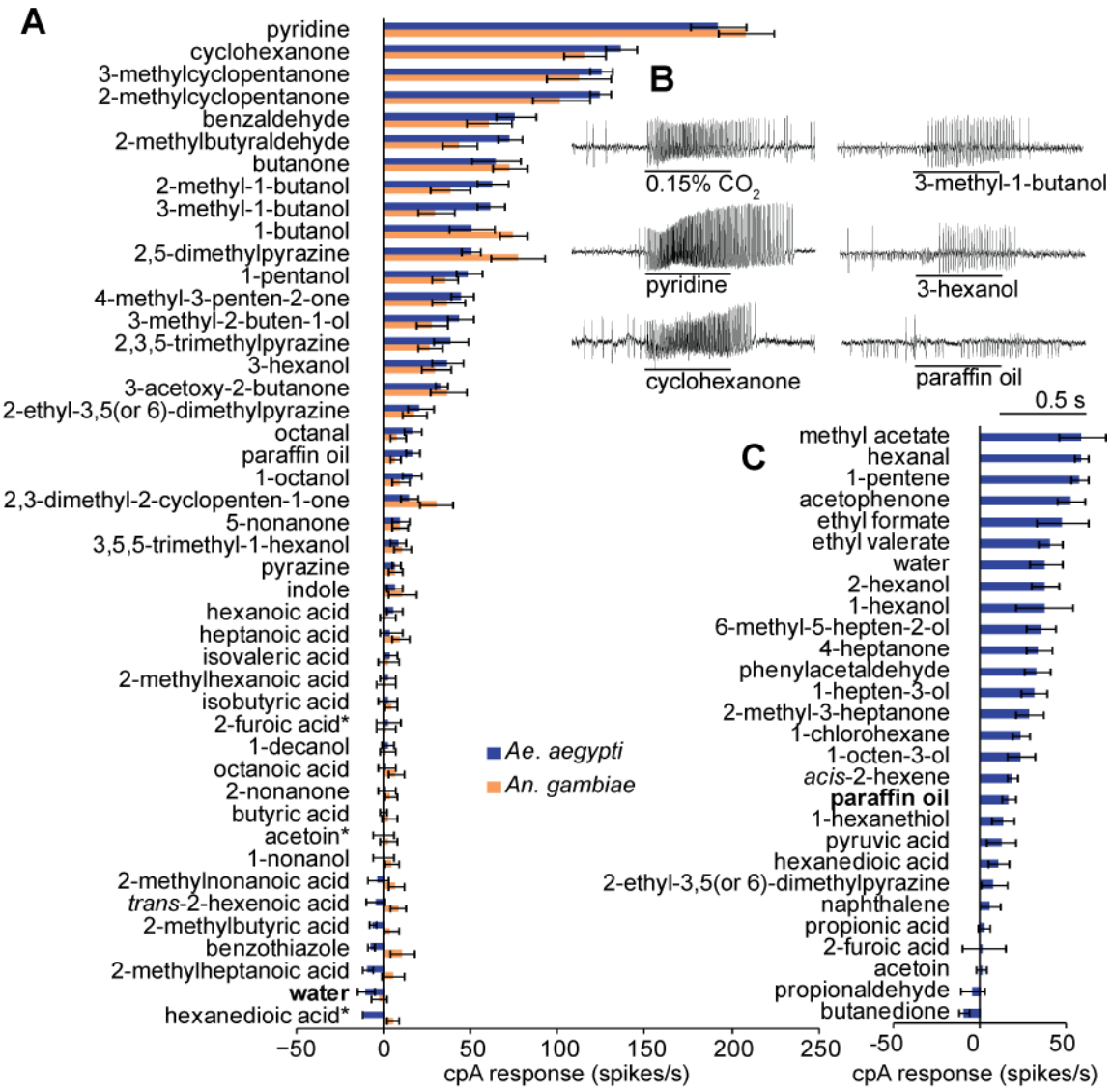


Figure 2.2. cpA detects many human-associated odorants.
(A) Mean responses of the cpA neuron to 0.5 s pulses of known components of human odor in *Ae. aegypti* and *Anopheles gambiae*. n = 2–17.
(B) Representative traces of *Ae. aegypti* cpA responding to odorants.
(C) Mean responses of cpA neuron to additional human odorants tested only in *Ae. aegypti*. n = 2–12.
All odorants were diluted in paraffin oil (PO) or *water at 10^{-2}. Error bars are s.e.m.
See Appendices 2 and 3 for details on odorants and cpA responses.

At the time this experiment was conducted, the *An. gambiae* colony was struggling and females were not always available; when they were available, they were not always in good health. CpA responses varied considerably between cohorts of mosquitoes, as shown in (Fig. 2.3); cpA responses in the mosquitoes tested in July were lower across the board, although the pattern of responses is the same. The results in (Fig. 2.2A) include all three batches of mosquitoes.

The cpA neuron's responses to skin-derived odorants are dose-dependent and comparable to its responses to much higher concentrations of CO_2 (Fig. 2.4A). Because these odorants are liquid at room temperature and dissolved at the stated concentrations in

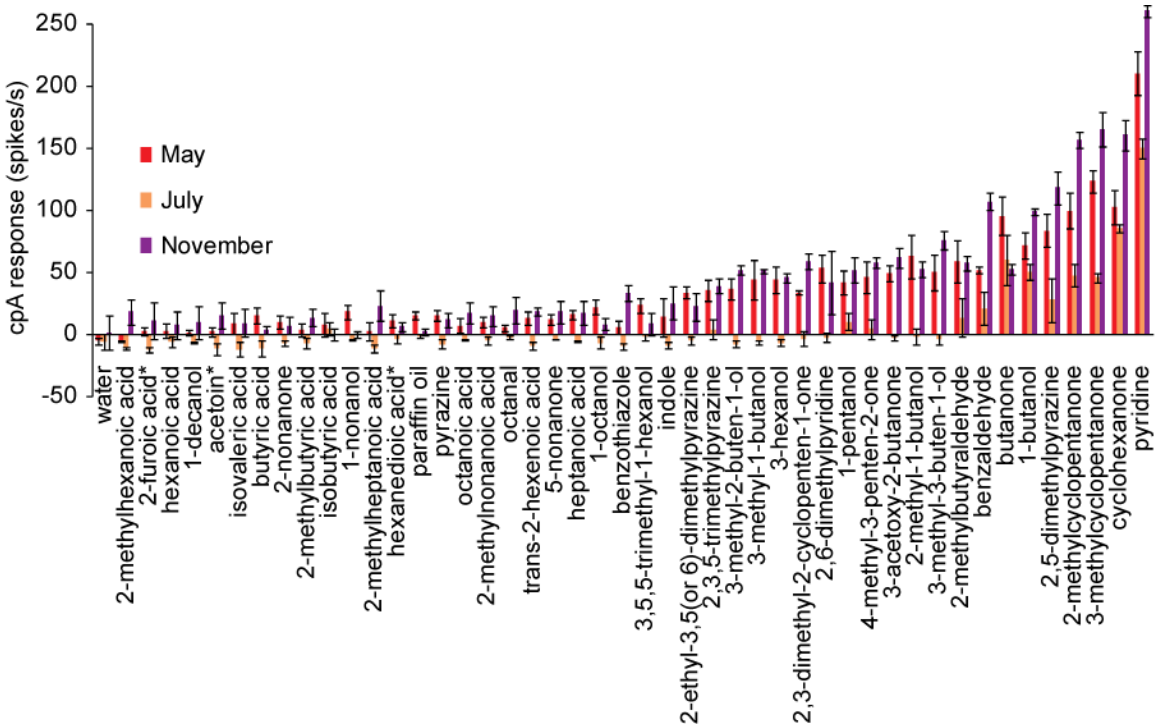


Figure 2.3. Variation in cpA responses observed in *An. gambiae*.
Mean cpA responses to a panel of odorants in *An. gambiae* mosquitoes reared at different times. CpA responses were similar in mosquitoes reared together. n = 3–4 per group. All odorants were diluted in paraffin oil or *water at 10^{-2}. Error bars are s.e.m.

paraffin oil, the amount of volatile chemical that reaches the insect is much lower than the stated concentration (Andersson et al. 2012). As CO_2 is delivered as a gas, it is diluted to the stated concentration in the airstream passing over the insect. The temporal kinetics of cpA's responses to these odorants are consistent across repeated stimulations, regardless of whether these are repeated every 15 s (Fig. 2.4B,C) or every 2 s (Fig. 2.5A). The kinetics of these odorant responses are also similar to those of CO_2 responses, with a clearly discernable phasic–tonic pattern in most cases (fig 2.3B,C; Grant et al. 1995). In its natural environment, a mosquito at a distance from a potential host will encounter plumes containing mixtures of CO_2 and skin odor at low concentrations. To test whether a mosquito would respond more sensitively to a combined stimulus, cpA responses to binary mixtures of the two types of activators (CO_2 and skin odorants) were measured. As long as neither stimulus alone would saturate the neuron's ability to respond, cpA's response to a mixture of CO_2 and skin odorant was significantly greater than its response to either stimulus alone, even when total airflow was kept constant (Fig. 2.5B). A similar trend was observed with odorants presented at a lower concentration ($10^{-3.5}$), although the differences were less clear due to low odorant responses (not shown). This overall result contrasts with *Or*-expressing neurons, where mixtures of two activating odorants do not elicit stronger responses than the stronger activator by itself (Münch et al. 2013). Prior exposure to CO_2 or skin odorants, however, did not change cpA responses to subsequent stimuli (Fig. 2.5C).

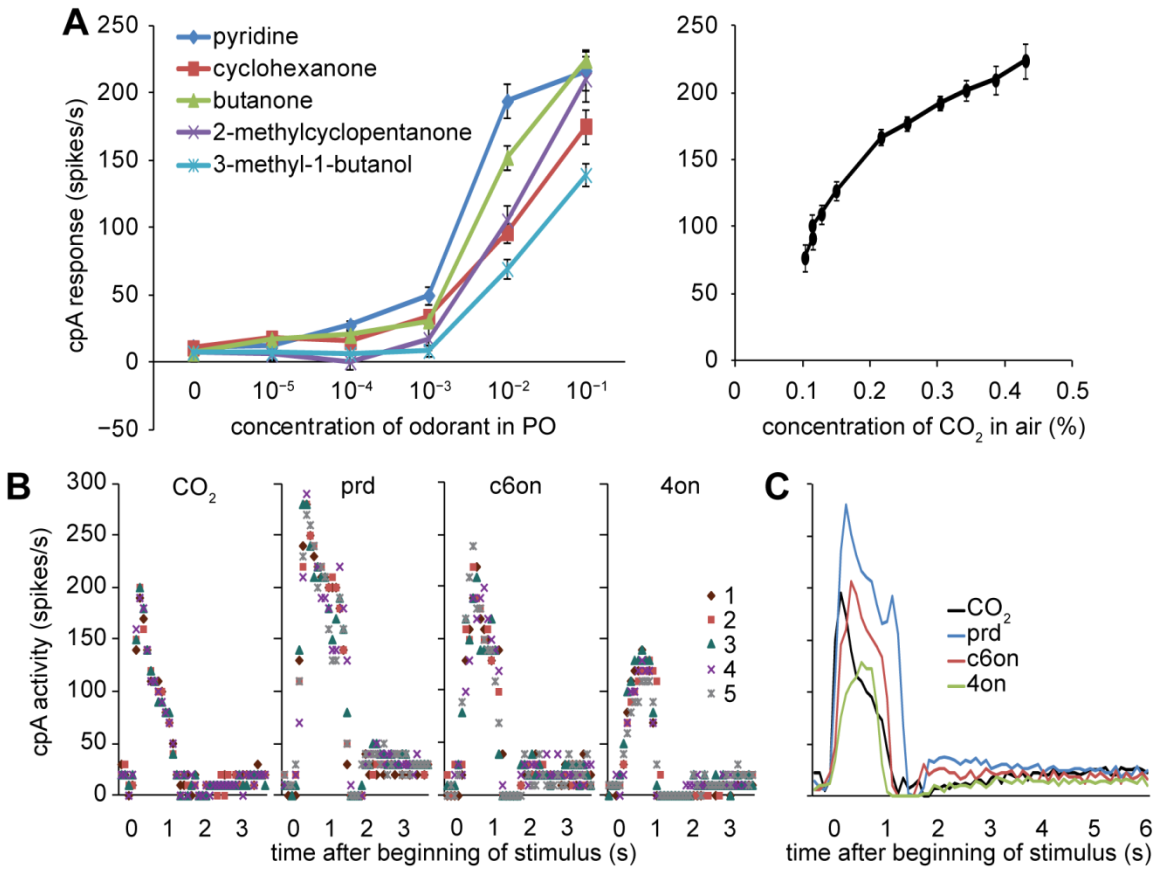


Figure 2.4. Properties of cpA responses to human-associated odorants.
(A) Dose responses to representative activating skin odorants (left), diluted in PO, and CO_2 (right), diluted in air. n = 5–6, error bars are s.e.m.
(B,C) Temporal response profiles of cpA activity elicited by 4–5 repeated 1 s pulses of indicated odorants spaced 15 s apart. Activity is shown per pulse (B) and averaged across pulses (C). CO_2 was diluted in air to 0.15%; other odorants were diluted in PO at 10^{-2}.
PO: paraffin oil. **prd:** pyridine. **c6on:** cyclohexanone. **4on:** butanone.

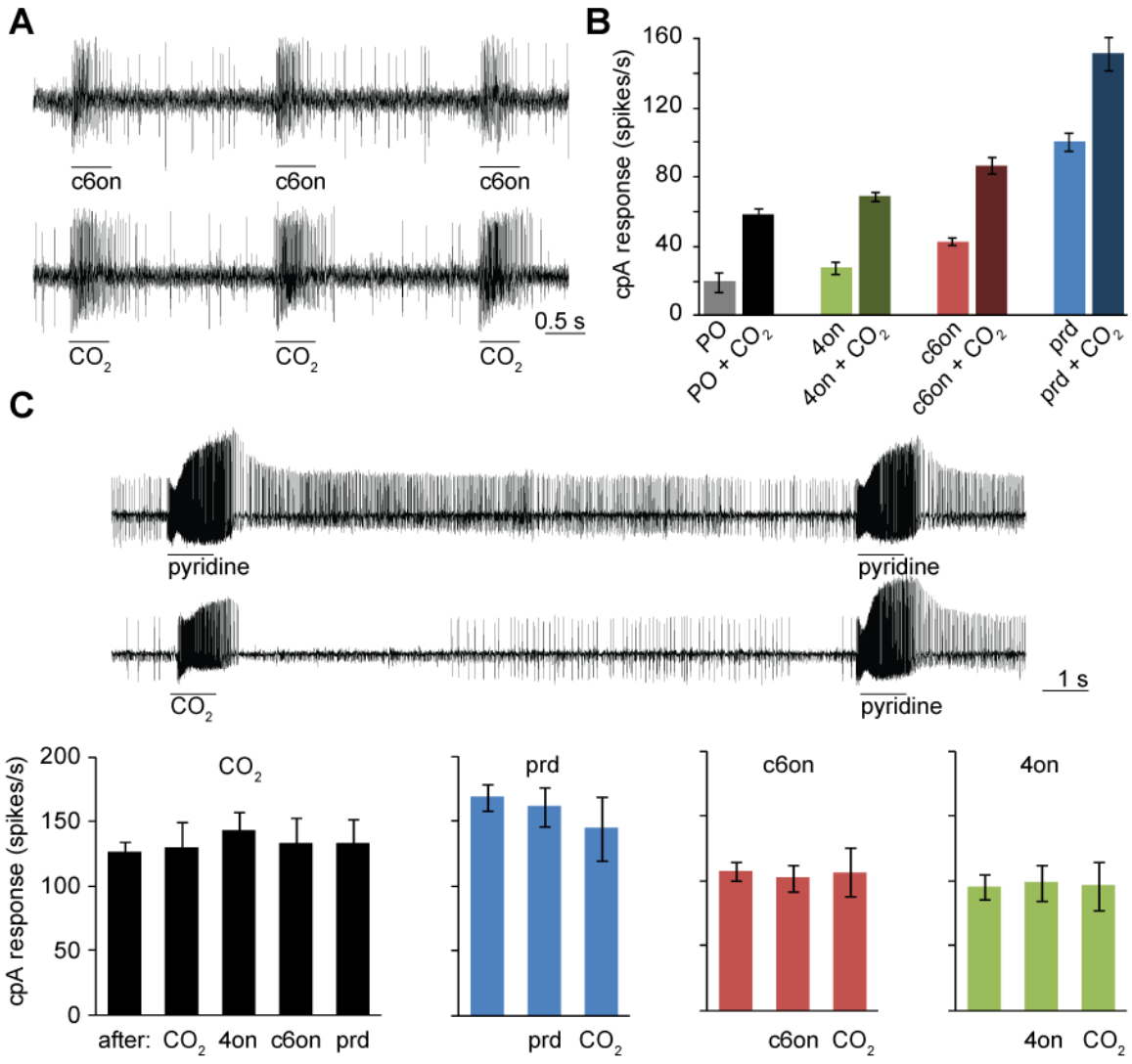
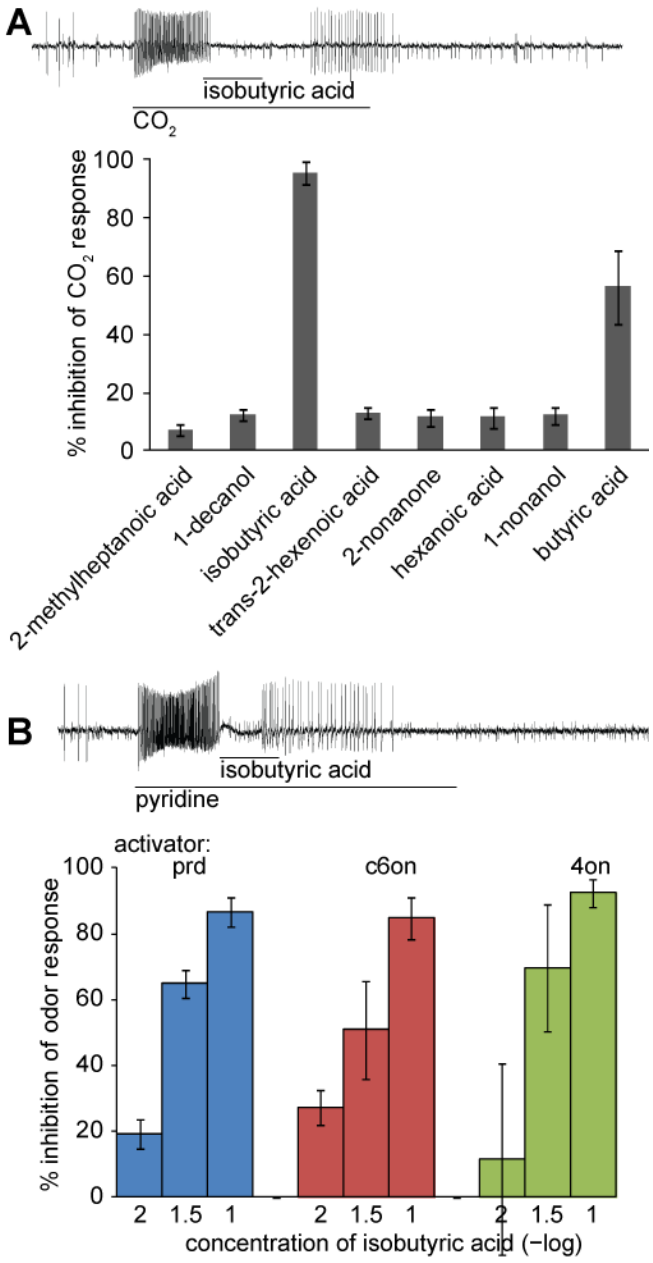


Figure 2.5. Interactions between cpA activators.
(A) Sample traces of cpA responses to repeated pulses of odorant (0.15% CO_2 or cyclohexanone at 10^{-2} in paraffin oil) spaced 2 s apart.
(B) cpA responses to combinations of skin odorants (at 10^{-3}) and 0.1% CO_2. Carrier airflow was adjusted between stimuli to maintain constant total airflow with and without added CO_2. $n = 6$.
(C) Sample traces and mean responses to activating odorants presented in 1 s pulses either first in a sequence (leftmost bar of each graph) or presented 15 s later. 0.15% CO_2, other odorants at 10^{-2} in paraffin oil. $n = 6$ replicates of each combination. There are no significant differences in responses to any odorant (ANOVA; $p > 0.05$).
prd: pyridine. **c6on:** cyclohexanone. **4on:** butanone. Error bars are s.e.m.



Two human-associated odorants are inhibitors of cpA

Eight compounds that did not evoke positive cpA responses in the initial screen of human odorants (Fig. 2.2) were screened for their ability to inhibit a positive stimulus of 0.1% CO_2. Two of these, butyric acid and isobutyric acid, noticeably inhibited cpA responses when they were introduced during a puff of CO_2 (Fig. 2.6A). This confirms a previous report that butyric acid is a potent inhibitor of CO_2 detection by cpA in both *Ae. aegypti* and *An. gambiae* (Turner et al. 2011). Additional tests were conducted with

Figure 2.6. Inhibitors of cpA from human odor.
(A) Representative trace and mean percent inhibition (compared to solvent) of cpA responses by odorants at 10^{-1} presented as a 0.5 s stimulus overlaid on a 2 s pulse of 0.1% CO_2. n = 5–6.
(B) Representative trace and mean percent inhibition (compared to solvent) of cpA responses by isobutyric acid presented at indicated concentrations as a 0.5 s stimulus overlaid on a 2 s pulse of indicated odorant. n = 6.
Error bars are s.e.m.

the stronger inhibitor, isobutyric acid, and found that it also inhibits responses to activating odorants in a concentration-dependent manner (Fig. 2.6B).

CpA detects whole human odor

In all of the above experiments, human-associated odorants were presented in isolation at high concentrations well above what might be encountered in the mosquito's natural environment. Additional experiments were conducted to determine whether cpA also detects the natural human odor blend at physiological concentrations. When air was puffed over a filter paper laden with human odor from GMT collected after 7 d without bathing, cpA responded strongly, at 49 spikes/s over the baseline firing rate (Fig. 2.7A).

Instead, I used an alternative method to collect human odor on glass beads. These beads are effective vehicles of human odor for chemical analysis and attract mosquitoes in laboratory assays (Bernier et al. 1999, Qiu et al. 2004, Lacey and Cardé 2011). Volunteers placed the beads in their clean socks and wore them for ~6 hr while carrying out normal daytime activities to coat the beads with foot odor. Human odor from the beads was used directly for electrophysiology, without use of solvents or any other concentration system. This was necessary because many polar solvents that would be most effective at dissolving the compounds of interest are actually cpA activators themselves, and cpA's response to the solvent effectively masks the response to the relatively small concentrations of dissolved odorants (personal observation). The solvent-free system has the added benefit of replicating the exact odor used in behavioral experiments (see below).

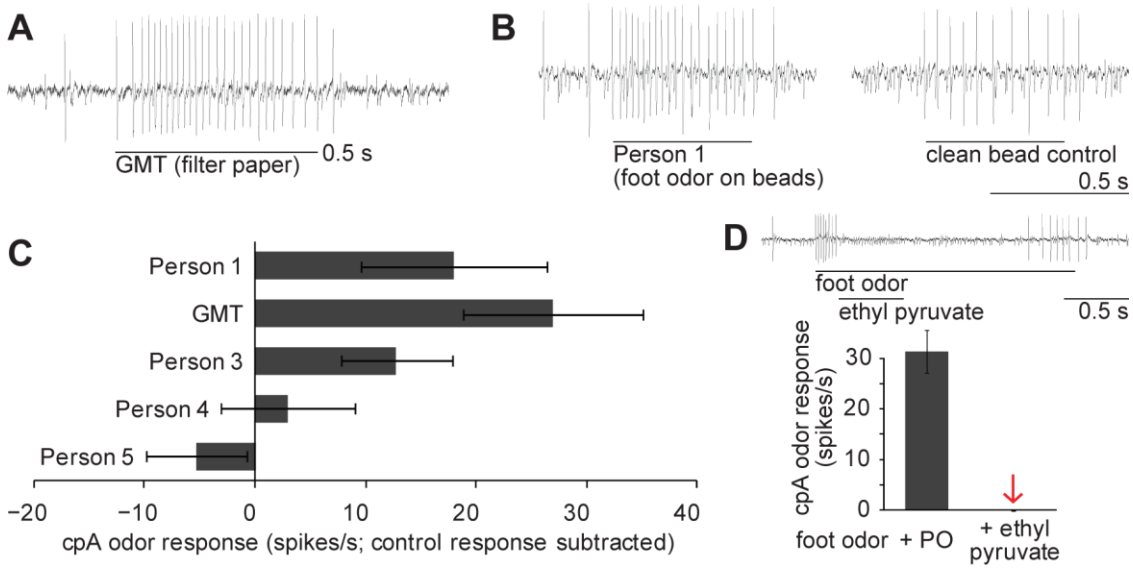


Figure 2.7. CpA detects whole human odor.
(A) cpA response to skin odor collected on filter paper after a 1 wk backpacking trip. (B) Representative traces and (C) mean change in cpA activity elicited by foot odor carried on glass beads. n = 4–10.
(D) Representative trace and mean cpA response during a 1 s stimulus of paraffin oil (PO) or ethyl pyruvate (10^{-2}) overlaid on a 2 s stimulus of foot odor (mixed beads from Person 1 and GMT). n = 6.

Odor from three of the five people tested consistently activated cpA above and beyond any mechanical artifact (Fig. 2.7B,C). This corroborates a previously unexplained observation that cpA activity increases when a human hand is placed nearby (Kellogg 1970). The amount of activation varied among odor donors, however, and odor from two other participants did not produce a measurable response.

As a side note, ethyl pyruvate was discovered by Dyan MacWilliam and Sean M. Boyle to be a potent inhibitor of cpA's CO_2 response with potential commercial application (Tauxe et al. 2013). This compound also completely inhibits cpA's response to the human odor blend (Fig. 2.7D).

Butyryl chloride is a long-term inhibitor of cpA

When I performed my first screen of compounds to look for additional cpA ligands, the chemicals I used were chosen solely on the basis of structural similarity to known ligands and commercial availability – that is, not for safety or stability. I found that over the course of presenting a panel of odorants, cpA would often stop responding to any of the tested odorants or even to exhaled breath (~4% CO_2), and that this phenomenon was associated with exposure to one of the test chemicals, butyryl chloride (diluted at 10^{-2} in paraffin oil). This lack of response often resembled a loss of signal from the sensillum, except that spikes from the co-sensillar neurons cpB and cpC were still visible (Fig. 2.8A). Puffs of butyryl chloride diluted at a tenfold higher concentration (10^{-1} in paraffin oil) induced not inhibition, but rather superactivation of cpA, along with insensitivity to CO_2 (not shown), similar to the effects of butanedione (Turner et al. 2011).

To achieve a consistent, complete inhibition of cpA by butyryl chloride, I developed a system to pre-expose a mosquito to volatile butyryl chloride before electrophysiological or behavioral testing. A 100 µl droplet of 1% butyryl chloride (by volume in paraffin oil) was pipetted onto the bottom of a glass dish, then the dish was upended on top of a glass surface and allowed to sit for 10–20 min so that the butyryl chloride could volatilize. A mosquito prepared on a slide for electrophysiology or in a release cage for behavioral experiments was introduced into the glass dish and left for 60–180 s (Fig. 2.7B). Frozen aliquots of 1% butyryl chloride can be used, but each one

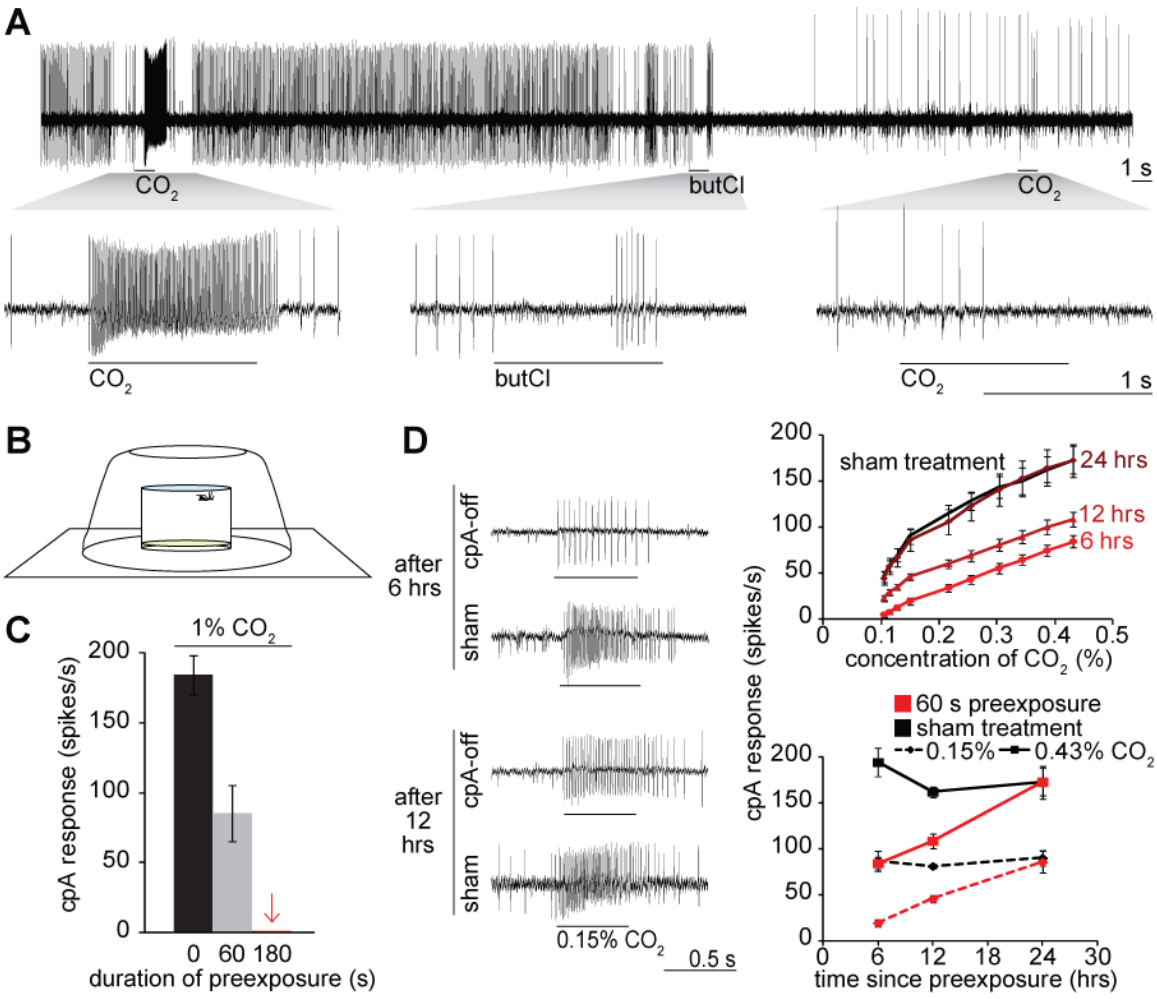


Figure 2.8. Effects of butyryl chloride exposure on CO_2 responses.
(A) Representative trace of cpA activity in response to 0.1% CO_2 before and after a 1 s exposure to butyryl chloride (10^{-2} in PO).
(B) Schematic of glass chamber used to treat mosquitoes with butyryl chloride.
(C) CpA responses to 1% CO_2 after preexposure to butyryl chloride using method illustrated in (B) for varying lengths of time.
(D) Representative traces and mean responses to varying concentrations of CO_2 hr after preexposure to butyryl chloride for 60 s. n = 10 across 4 individuals per condition.
butCl: butyryl chloride. Error bars are s.e.m.

can only be used for one treatment. For sham treatments, an identical setup is used except that paraffin oil is used instead of 1% butyryl chloride.

Using this system, I observed substantial inhibition of cpA's responses to CO_2 after 60 s of pre-exposure to butyryl chloride and complete inhibition after 180 s (Fig. 2.8C). CpA responses to a range of CO_2 concentrations were still substantially reduced 6 hr after a 60 s exposure and did not recover completely until between 12–24 hr after treatment (Fig. 2.8D).

While cpA did not respond at all to even high concentrations of CO_2 (such as the 1% stimulus used for quantitative measurement or the ~4% concentration in breath) after a 180-s pre-exposure to butyryl chloride, it did respond at a reduced level to other strongly activating odorants (Fig. 2.9A), suggesting that these odorants may interact with receptors in the cpA membrane through a different mechanism or binding site from CO_2. CpA inhibition caused by butyryl chloride exposure appears to occur at the receptor, because the receptor potential evoked by CO_2 is eliminated and that evoked by other odorants is dramatically reduced after treatment (Fig. 2.9B). CpA's response to foot odor is completely lost after treatment (Fig. 2.9C). I refer to mosquitoes that have been treated with butyryl chloride in this manner as "cpA–off."

Odor-evoked responses of the other two neurons in the same sensillum (cpB and cpC) were not reduced by the treatment (Fig. 2.10A). In fact, these neurons slightly increased in activity, possibly due to release of ephaptic inhibition between co-sensillar ORNs (Su et al. 2012). The relatively small spikes of these neurons are not easily distinguished in most single-sensillum recordings, so they are counted together in the

data presented here. CpC expresses Or8 (along with orco) and is homologous to cpB in *Culex quinquefasciatus* Say and *An. gambiae* (Lu et al. 2007, Syed and Leal 2007). This neuron is sensitive to 1-octen-3-ol (Bohbot and Dickens 2009); however, this ligand was not used because in this strain of mosquitoes it superactivates the neuron, even at very low concentrations (personal observation). CpC responses were observed to water, 3-hexanol, and 3-methyl-1-butanol. CpB appears to express Or49/orco, which does not have an equivalent on the *An. gambiae* palp, and has no known ligands (Bohbot et al. 2007, Lu et al. 2007). In this study it responded moderately to cyclohexanone, 3-hexanol, and 3-methyl-1-butanol. It is likely that additional ligands for this neuron have not been observed because they also activate cpA, which would effectively mask any cpB responses. The weak response of cpB and cpC to foot odor is not affected by butyryl chloride treatment (Fig. 2.10B).

The summed response of antennal neurons to foot odor or to a synthetic blend of human odorants, measured by electroantennograms (EAG), also did not change with butyryl chloride treatment (Fig. 2.10C). Antennal responses to individual skin odorants were unaffected by butyryl chloride pre-treatment (Fig. 2.10D,E), at least when odorants were at the relatively high concentration of 10^{-1} in paraffin oil. At 10^{-2}, responses to butanone and cyclohexanone were slightly, but significantly, reduced. Mosquito antennal ORNs are not well mapped, so it is impossible to say whether those reductions are due to a single class of ORNs with reduced sensitivity or due to some other factor. In a follow-up experiment, Dyan MacWilliam found that odorant responses in many antennal trichoid sensilla are largely unchanged after a 1 s butyryl chloride exposure (Tauxe et al. 2013).

It is possible that butyryl chloride could have a systemic effect, in addition to shutting off cpA, but this appears not to be the case. In the absence of a CO_2 stimulus, mosquitoes in a small cage will tend to rest at the top of the cage, especially when a source of heat and humidity is placed above it. This short-range attractive behavior does not depend on cpA and is unaffected by exposure to butyryl chloride (Fig. 2.10F). A pilot experiment also found no clear difference in mosquito longevity after butyryl chloride treatment compared to controls (not shown).

Butyryl chloride is a highly reactive compound that is structurally related to butyraldehyde and butyric acid, both known cpA inhibitors (Turner et al. 2011), but the acyl functional group is connected to a chloride – an excellent leaving group for nucleophilic acyl substitution reactions. Because of this, butyryl chloride will react readily with water, alcohols, amines, and other nucleophilic compounds, resulting in the butyryl group covalently bonded to the attacking nucleophile and the concomintant formation of hydrochloric acid. This suggests a possible mechanism for the observed long-term inhibition of cpA by this compound: it may fit into the same binding pocket on the gustatory receptor as butyraldehyde or butyric acid, but then may form a covalent bond with a nucleophilic side group of an amino acid residue there, inhibiting the receptor's function permanently. This hypothesis fits with the observed recovery of the neuron after 12 hr, which may represent turnover of new gustatory receptor proteins in the membrane. Butyryl chloride is less effective at inhibiting the homologous ab1C neuron in *D. melanogaster* than it is at inhibiting cpA in *Ae. aegypti*, which may be

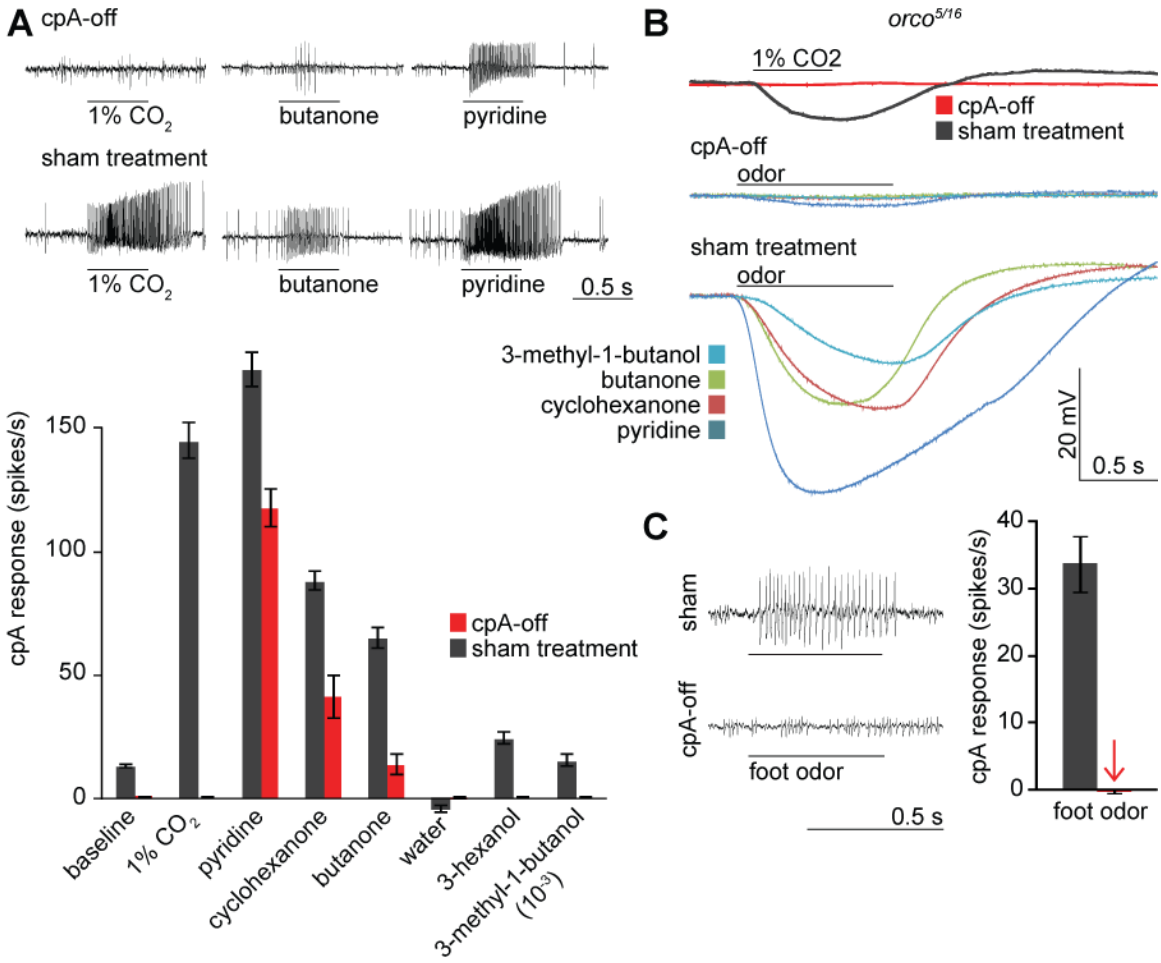


Figure 2.9. Effects of butyryl chloride exposure on responses to human odors.
(A) Representative traces (top) and mean cpA responses (bottom) to CO_2 and other human odorants after butyryl chloride treatment ("cpA-off") or sham treatment. *n* = 16 across 6 mosquitoes per treatment.
(B) Odor-evoked receptor potentials recorded from single sensilla after treatment. Recordings were made in *orco* trans-heterozygous mutant mosquitoes so that cpB and cpC do not contribute receptor potentials.
(C) Sample traces and mean responses to foot odor (mixed beads from Person 1 and GMT) after treatment. *n* = 8–9.
(A,C) Error bars are s.e.m.

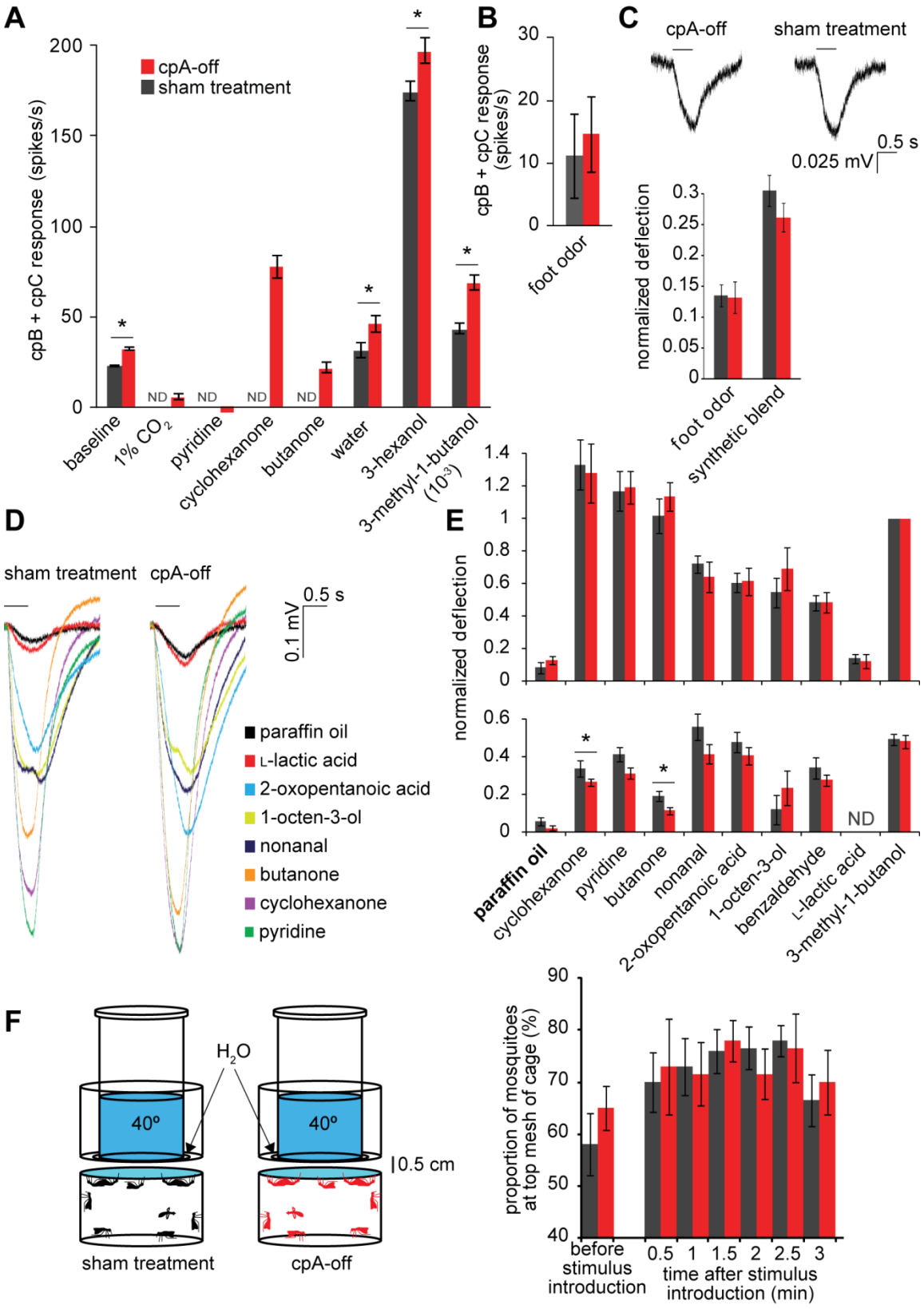

A
cpA-off
sham treatment
cpB + cpC response (spikes/s)
200
150
100
50
0
ND
baseline
1% CO2
pyridine
cyclohexanone
butanone
water
3-hexanol
3-methyl-1-butanol (10-3)
B
cpB + cpC response (spikes/s)
30
20
10
0
foot odor
C
cpA-off
sham treatment
0.5 s
0.025 mV
normalized deflection
0.3
0.25
0.2
0.15
0.1
0.05
0
foot odor
synthetic blend
D
sham treatment
cpA-off
0.5 s
0.1 mV
paraffin oil
L-lactic acid
2-oxopentanoic acid
1-octen-3-ol
nonanal
butanone
cyclohexanone
pyridine
E
normalized deflection
1.4
1.2
1
0.8
0.6
0.4
0.2
0
ND
paraffin oil
cyclohexanone
pyridine
butanone
nonanal
2-oxopentanoic acid
1-octen-3-ol
benzaldehyde
L-lactic acid
3-methyl-1-butanol
F
H2O
40°
40°
0.5 cm
sham treatment
cpA-off
proportion of mosquitoes at top mesh of cage (%)
90
80
70
60
50
40
before stimulus introduction
0.5
1
1.5
2
2.5
3
time after stimulus introduction (min)

Figure 2.10. Side effects of butyryl chloride treatment.
(A) Mean summed cpB and cpC responses to CO_2 and other human odorants after treatment. Differences tested by nested ANOVA; *$p < 0.05$. $n = 16$ across 6 mosquitoes per treatment. **ND**: no data.
(B) Mean summed cpB and cpC responses to foot odor (mixed beads from Person 1 and GMT) after treatment. Differences in odor responses are not significant (*t*-test; $p > 0.05$). $n = 4–8$.
(C) Averaged traces and mean normalized electroantennogram (EAG) responses to foot odor and to a synthetic blend of human odorants. $n = 16–18$ (foot odor), 8–9 (synthetic blend). Differences in odor responses are not significant (*t*=test; $p > 0.05$).
(D) Averaged traces and (E) mean normalized EAG responses to 0.5 s stimuli of indicated odorants. (E) Odorants at 10^{-1} (top) or 10^{-2} (bottom) in PO. Differences tested by *t*-test; *$p < 0.05$. $n = 9$.
(F) Schematic of apparatus used to assay short-range attraction to heat and humidity (left). Proportion of mosquitoes resting at the top surface of the cage before and after introduction of a warm, humid stimulus. Mosquitoes in each treatment group were observed to probe through the mesh with their proboscides when stimulus was present. No significant differences were found due to treatment (Mann-Whitney rank-sum test; $p > 0.05$). $n = 6$.

explained because butyric acid is also less effective at inhibiting this neuron (Turner et al. 2011, Tauxe et al. 2013).

CpA is required for behavioral activation by human odor

Initial behavioral tests with cpA–off mosquitoes found few obvious deficits in their ability to find host odor. (At the time of this study, receptor mutants were not available, so butyryl chloride treatment as described above was used to create mosquitoes with non-functional cpA neurons). The mosquitoes were capable flyers and found a human odor source (a dish of glass beads that had been worn in socks as described above) with no apparent difficulty. Some mosquitoes appeared not to activate, that is, they did not move during the assay, but because most of the mosquitoes tested were disturbed or knocked into flight by the opening of the release cage, this was not observed frequently.

To test possible effects on activation, the release protocol was modified to reduce the influence of potential activating stimuli other than the host odor itself. In particular, odors were not uncovered until the mosquito was at rest, i.e., did not move for at least 60 s or when the release cage was gently opened. Experimental parameters such as the distance of the odor source from the release cage (50~ cm) and the duration of the assay (5 min) were determined in pilot assays with untreated mosquitoes to maximize the observed difference in activation behavior downstream of odor-laden beads versus clean beads (Fig. 2.11A).

When sham-treated (control) mosquitoes were placed downwind of the human odor source in this experiment, most of them took off over the course of the 5-min assay, usually toward the beginning of the assay. Virtually all of these flew more or less

directly to the beads (Fig. 2.11C,D; Fig. 2.12). On landing, they probed avidly as if attempting to feed and stayed there until removed by the experimenter. In cases where the odor source was replaced with clean beads, most mosquitoes did not take off or move at all. Those that did flew around in the wind tunnel, apparently at random, sometimes coming to rest on the upwind mesh or the release apparatus but never on the beads (Fig. 2.11C,D; Fig. 2.12).

It became clear that cpA–off mosquitoes had a pronounced deficit in activation: in fact, most of those mosquitoes remained quiescent for the duration of the 5-min assay (or, in several cases when the assay was extended, >10 min). The proportion of mosquitoes tested that did take off at some point during the 5 min assay was indistinguishable from the proportion that took off without host odor (i.e., when the stimulus was replaced by a dish of clean beads). Three of the 7 mosquitoes that did take off flew directly to the beads, landed, and probed avidly until removed by the experimenter, just as observed with many sham-treated mosquitoes. The other 4 mosquitoes that took off did not interact with the beads but behaved more like sham-treated mosquitoes in the absence of host odor (Fig. 2.12). The basis for this apparently random difference in behavior is still unknown; it may be that the mosquitoes that navigated successfully to the beads were activated by an unidentified stimulus, or it may be that they took off as a random event, and subsequently encountered the host odor and turned upwind. In either case, it is clear that they were able to navigate toward, land on, and maintain probing in the odor-laden beads without functional cpA neurons. Thus, other chemosensory pathways are sufficient to mediate these behaviors.

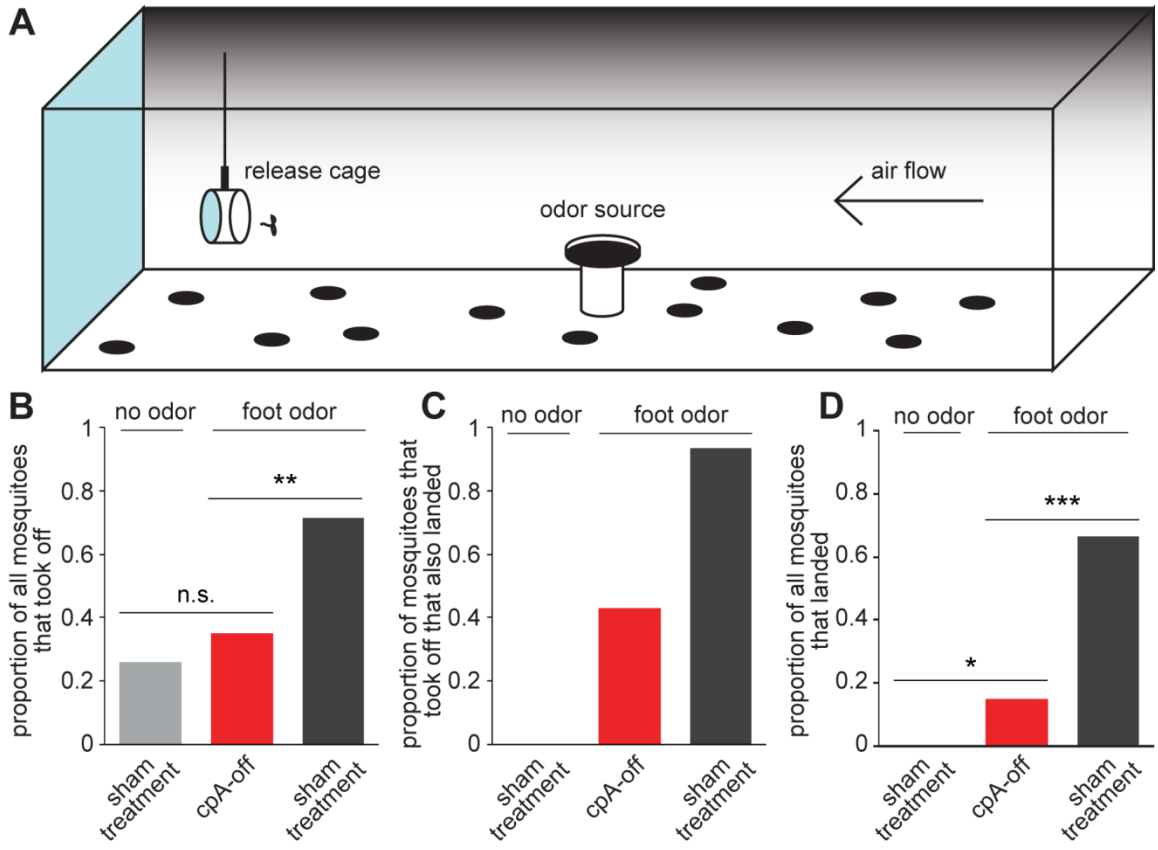


Figure 2.11. Role of cpA in odor-mediated activation and navigation toward a skin odor source.

(A) Schematic of wind tunnel assay for navigation of individual female *Ae. aegypti* to odor-laden glass beads. Dark circles along the bottom of the wind tunnel provide visual cues for flight.

(B) Proportion of cpA-off and sham-treated mosquitoes presented with clean or odor-laden beads that took off from the release cage, and (C) proportion of those mosquitoes that navigated to and landed on the glass beads.

(D) Proportion of all mosquitoes that navigated to and landed on the glass beads.

(B and D) n = 20–23 individuals. One-tailed proportion Z tests, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

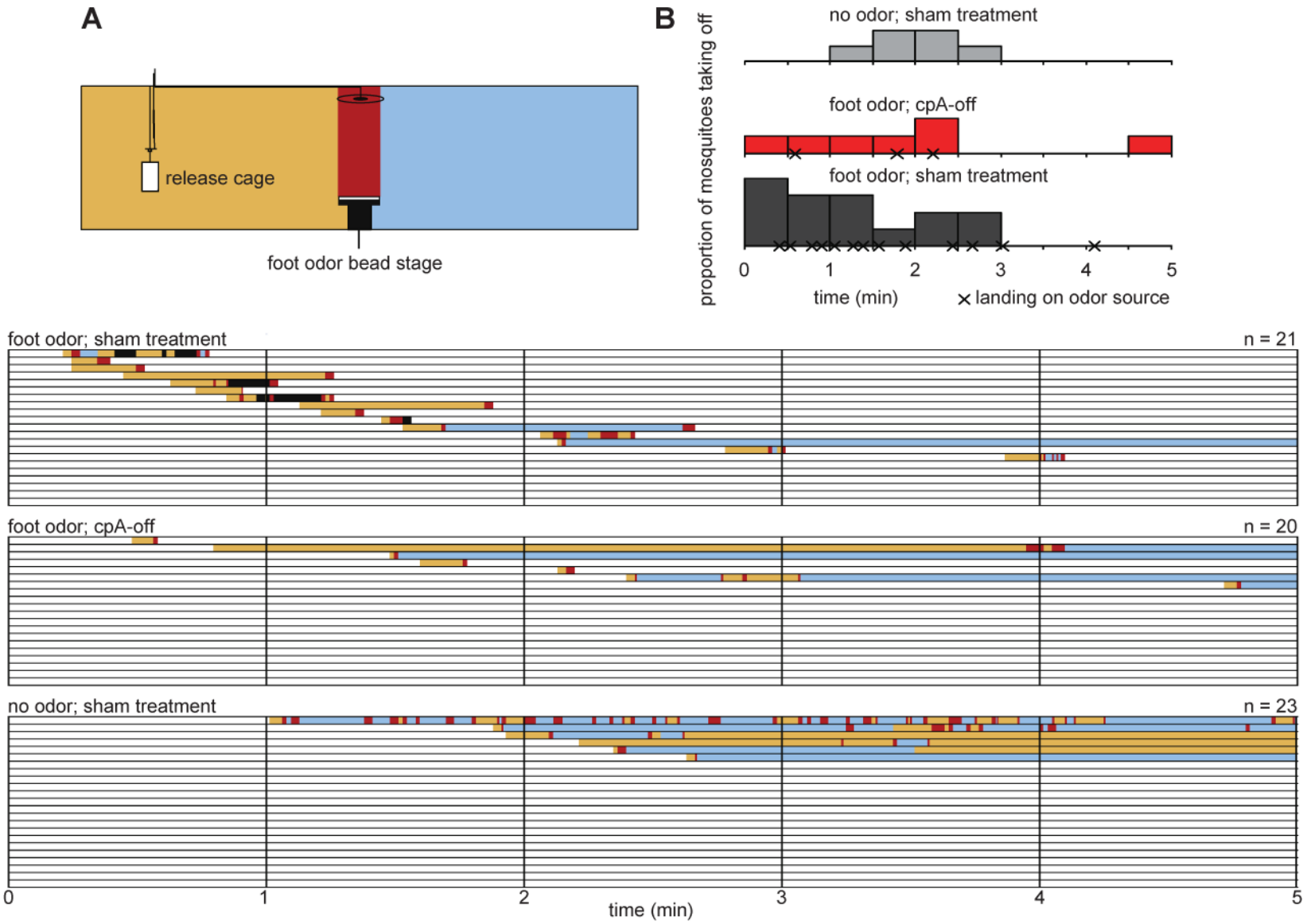

A
release cage
foot odor bead stage
B
no odor; sham treatment
foot odor; cpA-off
foot odor; sham treatment
proportion of mosquitoes taking off
0
1
2
3
4
5
time (min)
landing on odor source
foot odor; sham treatment
n = 21
foot odor; cpA-off
n = 20
no odor; sham treatment
n = 23
0
1
2
3
4
5
time (min)

Figure 2.12. Details of the wind tunnel assay.
(A) Schematic of the wind tunnel showing the mosquito release chamber, foot odor bead stage, and retracted bead cover.
(B) Histograms showing when mosquitoes took off during the 5 min assay in each of the three experimental conditions. Each × marks when a mosquito landed on the odor source.
(C) Coded mosquito activity during the assay. Each row indicates flight behavior of an individual mosquito. Shaded areas on each line correspond to time between when the mosquito left the release cage (if applicable) and when it landed on the beads or the assay ended. Colors correspond to the schematic in (A) and indicate in which region of the wind tunnel the mosquito was located at each moment.

Discussion

Prior to this work, the only published systematic studies which investigated whether non-CO_2 odorants might interact with cpA were by Lu et al. (2007) and Turner et al. (2011). The first study screened 97 structurally diverse odorants and found 3 that evoked increased firing compared to a solvent control (Lu et al. 2007). Turner et al. screened small aliphatic molecules with 4–8 carbons and found a small number of inhibitors that reduced cpA responses to CO_2 when they were presented together. This work breaks new ground by showing that, first, cpA responds to a much broader range of ligands than previously suspected, and second, that new ligands can be predicted based on structural similarity to known ligands and presence in an ecologically relevant blend. These results were used by Sean M. Boyle as training data for computationally predicting many new ligands, several of which have been shown to have interesting and potentially useful effects on mosquito behavior (Tauxe et al. 2013).

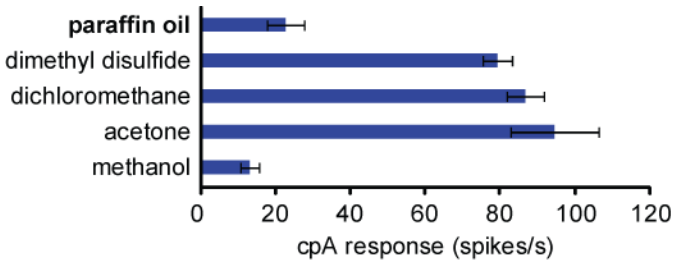


Figure 2.13. CpA detects odorants that mimic CO_2 in behavioral assays.
Mean cpA responses to 0.5 s pulses of odorants discovered by Bernier et al. (2003, Syed and Leal 2007) to evoke similar behaviors in *Ae. aegypti* as CO_2. Methanol was used in their study as a non-attractive control.
All odorants were diluted in (PO) at 10^{-2}. Error bars are s.e.m.

This also resolves the question of why mosquitoes are activated by whole human odor, but not by attractive components like L-lactic acid or 1-octen-3-ol: other components of the blend act through cpA to activate the

mosquito just as CO_2 does. Even though other neurons certainly detect human odor and are sufficient to allow a mosquito to find a host odor source, cpA is required for activation by odor. This may also explain the finding by Bernier et al. (2003) that the odor of three solvents mimicked the action of CO_2, activating *Ae. aegypti* and synergizing with L-lactic acid: all three of these compounds are strong cpA ligands (Fig. 2.13). Although cpA detects human-associated odorants more sensitively than CO_2 on a molecule-for-molecule basis, the very high concentrations of CO_2 in exhalations and that are used in traps mean that in ecologically relevant contexts, CO_2 is a much stronger stimulus than human odor. This is reflected in its much lower latency in activating mosquitoes, especially in turbulent plumes (Geier et al. 1999a, Dekker et al. 2005, Dekker and Cardé 2011). CO_2 is not sufficient to induce mosquitoes to land or enter traps, however (Gillies 1980, Cooperband and Cardé 2006), so these responses to human odor are likely mediated by human odor–sensitive ORNs other than cpA. This is supported both by my observation that cpA–off mosquitoes were capable of navigating toward and landing on a human odor source in the wind tunnel (Fig. 2.11C) and by another study showing that mosquitoes lacking functional cpA due to a mutation in *Gr3* were impaired at finding a live host in a large cage but not in a small cage (McMeniman et al. 2014).

The expanded ligand space of compounds which cpA detects includes a surprising diversity of chemical structures: everything from tiny CO_2 to short-chain carboxylic acids and ketones to relatively large, substituted heterocyclic aromatics. It is implausible that these compounds are all detected at a single receptor binding site. Indeed, the

observation that butyryl chloride treatment effectively inhibits cpA responses to CO_2 at even very high concentrations while it only partially inhibits responses to other odorants implies that CO_2 and other odorants are detected in at least two different binding sites. The mosquito CO_2 receptor consists of the three gustatory receptors Gr1, Gr2, and Gr3 in unknown stoichiometry. The *Drosophila* homolog has only two gustatory receptors, but an apparently similar breadth of ligands (Tauxe et al. 2013). In the next chapter, I will investigate how each of the mosquito receptors contributes to detection of different ligand classes.

Methods

Mosquitoes

Aedes aegypti. Most mosquitoes used were from the Rockefeller ("ROCK") strain, a laboratory strain colonized in Cuba before 1926, probably in 1881 (Kuno 2010). In order to eliminate odor-evoked responses from the cpB and cpC neurons, *orco* mutant mosquitoes were used for the receptor potential experiment (Fig. 2.9B). Mutant mosquitoes were heterozygous for two loss-of-function alleles of *orco*, $orco^{5}$ and $orco^{16}$. These mutants were created in an Orlando background and provided by the L. B. Vosshall laboratory (Rockefeller University; DeGennaro et al. 2013). Orlando strain mosquitoes were used as a wild type control; this is another selected laboratory strain that was probably established in Orlando, FL, in 1939 or 1942 (Kuno 2010).

Ae. aegypti of all strains were reared at 27°, 70% relative humidity, and L:D 14:10. Larvae were fed on alfalfa pellets; adults were fed 10% sucrose solution.

Colonies were maintained by females bloodfed on restrained mice or bovine blood provided through a membrane feeder. Females used in experiments were housed with males and were not bloodfed.

Anopheles gambiae. Mosquitoes of the G3 strain (MR4, Atlanta) were provided courtesy of M. C. Wirth and the W. E. Walton laboratory (UCR). They were reared in the Walton laboratory at 27–30°, 50% relative humidity and L:D 16:8. Larvae were fed Tetramin Tropical Fish Flakes ground into a fine powder; adults were fed 10% sucrose solution. Colonies were maintained by females bloodfed on restrained mice. Females used in experiments were housed with males and were not bloodfed.

Odors and stimulus presentation

Chemicals were obtained at the highest purity commercially available, typically >98% (Sigma-Aldrich), and dissolved in paraffin oil or water to the indicated concentrations (volume/volume for chemicals that are liquid at room temperature, or weight/volume for chemicals that are solid at room temperature). Odor cartridges were constructed for electrophysiology by applying 50 µl of dissolved odorant onto the cotton plug of a 5¾" Pasteur pipet capped with a blue tip and sealed with Parafilm between uses. Stimuli were presented in the same order across replicates of large odor panels; each cartridge was used for ≤3 stimuli. A constant 5–7 ml/s stream of carbon filtered room air was switched from a blank cartridge to the odor cartridge using a Syntech CS-55. The resulting airflow was delivered into a glass tube with a constant, humidified airstream (10 ml/s) whose mouth was centered on and ~1 cm from the insect head. CO_2 stimuli were pulsed using a PM8000 microinjector (MicroData Intrument, Inc.) to deliver controlled

pulses from pressurized cylinders of 1% or 5% CO_2 in air into the same carrier airstream, resulting in the indicated final concentrations of gas at the insect head. To measure responses to binary mixtures of CO_2 and activating odorants, the carrier airflow rate was adjusted to keep total airflow constant between conditions with and without CO_2.

Human odor. Odor was collected for the recording in Fig. 2.7A on clean filter paper held to the shin (GMT) with clear plastic wrap for 40 min after a 7 d backpacking trip without bathing. Odor-laden filter paper was stored at −20°C for 11 days. A ~0.5 cm × 2 cm strip of odor-laden paper was inserted into a flint glass Pasteur pipet and capped with a blue tip to construct an odor cartridge used as above for electrophysiology.

For other experiments, human odor was collected on glass beads worn in socks. Volunteers inserted 10–13 ml craft beads (size 10/0; Michaels) into each of their socks so that the beads were brought into contact with the toes and sole of the foot for ~6 hr of normal activity. To reduce extraneous odors, volunteers washed their feet with fragrance-free soap (Dove) and water immediately before odor collection, and socks were laundered between uses with fragrance-free detergent (Tide). Beads were cleaned between uses by agitation in a sonication bath with deionized water and Micro-90 detergent, thorough rinsing with deionized water, rinsing with distilled or HPLC-grade acetone, and heating to ~250°C for >4 hr. For electrophysiology, 20 ml beads were placed inside a 25 ml disposable serological pipet capped with a blue tip and sealed with Parafilm between uses. A constant 8 ml/s stream of carbon filtered room air was switched from a comparable cartridge with clean beads to the odor cartridge using a Syntech CS-55, as described above for stimulation with chemical odorants. Control

responses to clean beads were subtracted from the results reported; cpA neurons with >20 spikes/s response to a control puff were not considered.

Synthetic human odor blend. A synthetic blend of human-associated cpA activators was created based on the relative abundance of five compounds reported by Meijerink et al. (2000). This blend consisted of 1-pentanol, 3-methyl-2-buten-1-ol, pyridine, 4-methyl-3-penten-2-one, and cyclohexanone in a 3:3:3:2:2 ratio, all diluted at 10^{-3} in paraffin oil.

Electrophysiology

Single-sensillum recordings. Adult female mosquitoes (3–12 days old) were used for recordings. Each mosquito was restrained on a microscope slide with its head propped up and stuck to an elevated cover slip with double-stick tape (3M). The maxillary palp was gently brushed with tape to remove scales and pressed into tape on the cover slip with a blunt glass needle to keep it from moving. A reference electrode, consisting of either a silver wire electrode inserted into a glass micropipet filled with sensillum lymph ringer (Kaissling and Thorson 1980) or a fire-sharpened tungsten electrode, was inserted into the insect's eye. A recording electrode, consisting of a silver wire electrode inserted into a glass micropipet filled with sensillum lymph ringer, was inserted into the base of a capitate peg sensillum using a micro-manipulator under magnification.

Signals were amplified 1000× and band-pass filtered to admit signals between 10 Hz–1.0 kHz with a Iso-Dam amplifier (World Precision Instruments). Signals were digitized with a Digidata 1440 (Molecular Devices). Recordings were analyzed in the

AxoScope and pClamp programs (Molecular Devices); spikes (i.e., action potentials) were counted manually or in the Igor Pro 6.2 program (Wavemetrics) with the Neuromatic v2.00 macro by Jason Rothman. Neuronal responses were corrected for baseline firing rate: reported firing frequencies were calculated as 2 × (number of spikes during first 0.5 s of stimulus presentation) – (number of spikes during 1 s prior to stimulus presentation). Percent inhibition was calculated relative to the response to a solvent control in the same sensillum. Firing frequency was calculated in 100 ms bins to describe temporal dynamics of responses (Fig. 2.4B,C).

Receptor potentials. Sensillar receptor potentials were recorded as above except that signals were amplified 100× and band-pass filtered to admit signals between 0.1 Hz–10.0 kHz.

Electroantennography (EAG). Decapitated heads of adult female mosquitoes (4–14 days old) were used for recordings. A reference electrode consisted of a silver chloride–coated silver wire inserted into a glass capillary filled with Beadle-Ephrussi ringer (Benton and Dahanukar 2010). The capillary was sealed at the distal end with a pore blown in the side, into which the neck tissue of the mosquito head was inserted. The tip of the antenna was removed using a scalpel to cut through the distalmost flagellomere and a recording electrode, consisting of a silver chloride–coated silver wire inserted into a saline-filled micropipet, was placed over the cut end.

EAG signals were amplified 100× and band-pass filtered to admit signals from 0.1 Hz–10.0 kHz. Maximum deflections evoked by odor stimuli were normalized to interspersed pulses of a reference odorant (3-methyl-1-butanol diluted at 10^{-1} in paraffin

oil) according to the formula: Raw response (mV)/($ax + by$), where a and b are the responses (mV) to the previous and subsequent reference odor stimulation, respectively, and x and y are the proportion of time elapsed between stimuli (so that $x + y = 1$). Responses to the reference odorant did not differ between treatment groups.

Chemical genetics

Mosquitoes were individually pre-treated with butyryl chloride by placing the release cage (for behavior) or slide-mounted mosquito (for electrophysiology) in an upended 1 qt (0.47 L) glass dish in which 100 μl butyryl chloride diluted at 10^{-2} in paraffin oil (treatment/cpA–off) or paraffin oil (sham treatment) had been allowed to volatilize at room temperature for 10–20 min. Dishes were cleaned with ethanol, dried with a KimWipe, and a fresh application of chemical was used for each treatment. Aliquots of butyryl chloride dissolved in paraffin oil were mixed fresh and stored at −20° to maintain purity.

Behavior

Wind tunnel. Behavior experiments were performed in a glass wind tunnel with dimensions 36 cm × 40 cm × 128 cm in a room illuminated with fluorescent overhead lights. Room air (27°C, 35–40% relative humidity) was carbon filtered and drawn through the wind tunnel in a laminar flow at a constant rate of 0.2 m/s. Mosquitoes were introduced in a release cage made of Plexiglas tubing with screening glued over one end and a slit towards the other end through which a removable manila card was inserted to block mosquitoes from exiting. Odor was provided in the form of 20 ml odor-laden or

clean control beads in a covered 10 cm diameter petri dish elevated 7 cm above the floor of the wind tunnel 50 cm directly upwind from the release cage holder (Fig. 2.11A). The wind tunnel was thoroughly cleaned with 70% ethanol before and between experiments, and all equipment used in the wind tunnel was handled while wearing nitrile lab gloves and discarded or washed with fragrance-free detergent (Tide) each day to reduce odor contamination.

8–14-day-old non-bloodfed female *Ae. aegypti* were held in individual release cages without access to food or water for 17–23 hr at 27°C and ~70% relative humidity before testing. Each mosquito was pre-exposed for 180 s to butyryl chloride or solvent immediately before testing. When introduced into the wind tunnel, the manila card was removed and the mosquito was prevented from exiting the release cage by placing a mesh flap over the open end until she was still for at least 60 s within 4 min of being placed in the wind tunnel. At that point, covers were removed from both the beads and the release cage exit to start the assay, which was video recorded for 5 min or until the mosquito landed on or walked onto the beads. Activation was defined as leaving the release cage; in every case the mosquito left by flying out. Landing was defined as alighting on the beads or alighting elsewhere on the stimulus apparatus and walking onto the beads. Landing was followed in all cases by probing with the proboscis. Trials were conducted from 14:00–18:30. On days when control mosquitoes did not approach the odor source, the odor was deemed insufficiently attractive and data were not considered.

Short-range attraction. Ten 6-day-old female *Ae. aegypti* were starved 30 hr in a 7 cm diameter × ~5 cm high cage with wire mesh on one side and closed by a manila

card on the opposite side. Test cages were placed inside a 10 gal (38 L) aquarium and left undisturbed 5 min, after which a filter paper soaked with 400 ml water and a beaker containing 750 ml 40°C water were placed 5 mm above each cage (Fig 2.10F). Mosquito behavior was then video recorded for 3 min.

Statistics

All statistical analyses were performed in R. Except where noted, electrophysiological data were recorded with one replicate per insect.

Ethics

Use of animals for feeding mosquitoes was monitored by the Institutional Animal Care and Use Committee at UCR and conducted in accordance with protocol A-2010023 issued to Ring Cardé and Anandasankar Ray.

Chapter 3. Functional analysis of subunit interactions in the insect carbon dioxide receptor.

Insect olfactory receptors include the odorant receptors (ORs), the ionotropic receptors (IRs), and one small group of gustatory receptors (GRs), the Gr1,2,3 clade. Most GRs are involved in taste, but these three GRs are expressed together and confer sensitivity to carbon dioxide (CO_2) (Jones et al. 2007, Kwon et al. 2007, Lu et al. 2007). This clade is highly conserved across holometabolous insects except Hymenoptera, with one curious exception: there is no homolog for Gr2 present in *Drosophila* (Robertson and Kent 2009). It is not known how Gr1, Gr2, and Gr3 interact to form a functional unit.

It has recently been shown in mosquitoes that the GR-expressing, CO_2-sensitive cpA class of neurons also detects a wide variety of other compounds (Chapter 2, Tauxe et al. 2013). These additional odorants are not characterized by any particular functional group and include short aliphatic compounds, cyclic aliphatic compounds, and aromatic heterocyclics. Some compounds that inhibit the CO_2 response are structurally related to activators and may act via the same binding site. A newly identified long-term inhibitor, butyryl chloride, completely inhibits cpA neurons from responding to CO_2 but only partially inhibits its responses to other odorants, suggesting that the heteromeric receptor may have at least two different odorant binding sites (Chapter 2).

GRs are distantly related to ORs and together they make up the insect chemoreceptor superfamily. Proteins in both families are membrane bound with seven predicted transmembrane domains. ORs have a reversed topology compared to G

protein–coupled receptors (GPCRs), with their N-terminus inside the cell (Benton et al. 2006, Lundin et al. 2007, Smart et al. 2008), as do taste GRs (Zhang et al. 2011, Xu et al. 2012). ORs can interact with G proteins *in vitro*, but there is little evidence that this is a functional signaling pathway *in vivo* (Wicher et al. 2008, Yao and Carlson 2010). Rather, current evidence suggests that a odorant-specific tuning OR forms a heteromer with the obligatory OR co-receptor orco to form a ligand-gated cation channel (Neuhaus et al. 2005, Benton et al. 2006, Sato et al. 2008, Jones et al. 2011).

There is more evidence that GRs may use a G protein signaling pathway, perhaps in addition to an ionotropic response. Mutations in the signaling proteins $G\alpha_q$, $G\alpha_o$, and IP3 all impair detection of various subsets of sugars in *Drosophila melanogaster* Meigen, 1830 (Usui-Aoki et al. 2005, Bredendiek et al. 2010, Kain et al. 2010). The olfactory CO_2 response is also partially dependent on a $G\alpha_q$ pathway. RNAi knockdown and a competitive peptide agonist of $G\alpha_q$ both strongly reduce the response of *Drosophila* ab1C neurons to CO_2 (Yao and Carlson 2010).

Very little is known about how GRs in the same cell interact. No orco-like co-receptor has yet been identified, but multiple GRs are usually expressed together in both the taste and olfactory systems, so a functional unit may require more than one type of GR. The *Drosophila* CO_2 receptor consists of two members of the Gr1,2,3 clade, DmGr21a and DmGr63a, which are co-expressed in the ab1C neuron on the antenna. If DmGr63a is not present, the neuron no longer responds to CO_2 (Jones et al. 2007) or to other classes of odorants (Tauxe et al. 2013). Likewise, in recently created DmGr21a mutants, ab1C no longer responds to CO_2 (S. Perry, personal communication).

The original empty neuron system was developed at the Carlson laboratory (Yale University) to decode ORs in a heterologous neuron (Dobritsa et al. 2003, Hallem et al. 2004). This system relies on the *Δhalo* deletion mutant, which lacks both DmOr22a and DmOr22b receptors normally expressed in ab3A antennal neurons. Other ORs can be expressed in the empty ab3A neuron using the GAL4–UAS system and confer their own odor specificities. This versatile system has been used to decode OR repertoires from *Drosophila* and also the mosquito *Anopheles gambiae* Giles, 1902 (Hallem and Carlson 2006, Carey et al. 2010).

When the *Drosophila* olfactory GRs DmGr21a and DmGr63a are expressed in this system, neither receptor alone is sufficient to confer a CO_2 response. The combination of both forms a functional receptor weakly responsive to CO_2 (Jones et al. 2007, Kwon et al. 2007). Some combinations of the *An. gambiae* homologs have been observed to confer a weak CO_2 response in the same system (Lu et al. 2007). CO_2 responses were weak in this system presumably because this normally OR-expressing neuron lacks some cellular machinery that facilitates GR function. Indeed, co-expressing $G\alpha_q$ with DmGr21a and DmGr63a in this system improves the sensitivity of the transgenic neuron to CO_2 by a factor of five (Yao and Carlson 2010).

The recent creation of a *DmGr21a* mutant fly by Sarah Perry in the Ray Laboratory (UCR) has made it possible to create a new empty neuron in the natively GR–expressing ab1C. To investigate the functional roles of the three subunits of the insect CO_2 receptor, I cloned *AaGr1*, *2*, and *3* from the mosquito *Aedes aegypti* (L., 1762) and created transgenic flies that express them under the control of the GAL4–UAS expression

system. I have expressed these receptors in the natively GR-expressing ab1C neuron on the *D. melanogaster* antenna in various combinations both with and without the native *Drosophila* GRs. Here I report on these investigations into how the three receptor subunits interact to respond to different classes of odorants.

The cloned gustatory receptor genes *AaGr1*, *Gr2*, and *Gr3*

AaGr1 and *AaGr3* were cloned using the pENTR/dTOPO cloning system (Invitrogen). This system was not successful for cloning *AaGr2*, so an alternative method developed by G. M. Pask called pATTL was used (see Methods). Clones in pUASg-attB-DV destination vector plasmids were injected into *Drosophila* embryos for site-directed transgenic insertion using the ΦC31 system. To facilitate testing combinations of transgenes, three sites were selected: one on chromosome II and two on chromosome III. Individual transgenic flies marked by mini-white were used to generate isogenic lines, and presence of the UAS–AaGr construct was confirmed by PCR.

The sequence of the *Ae. aegypti* receptor genes *Gr1*, *Gr2*, and *Gr3* and their translations have been published previously (VectorBase , Robertson and Kent 2009). Two highly similar alleles of *AaGr1* were detected in the colony of Orlando strain mosquitoes from which the gene was cloned; they differ by 5 SNPs, all but one of which are silent mutations. The cloned sequence of *AaGr1* differs from the published sequence at 11 SNPs, two of which are sense mutations; one of these is polymorphic across the two detected alleles in the laboratory colony.

Published sequences of AaGr2 from VectorBase and Robertson and Kent (2009) differ: the VectorBase sequence is longer by 25 residues at the N terminus, resulting from a 75–base pair extension of the open reading frame at the 5′ end of *Gr2*. This 75-bp region is not conserved in other mosquito species. PCR with primers targeting different regions of this gene indicates that mRNA with the 5′ extension was expressed in the *Ae. aegypti* population used for cloning, but at a lower frequency than without the extension (Fig. 3.1). The cloned sequence of *AaGr2* matches the VectorBase published sequence, but lacks the 75-bp 5′ extension.

Two separate clones of *AaGr3* were generated that differ by 10 SNPs; the sequence closer to previously published sequences was selected for use. This clone differs from the published sequence at 20 SNPs, three of which are sense mutations.

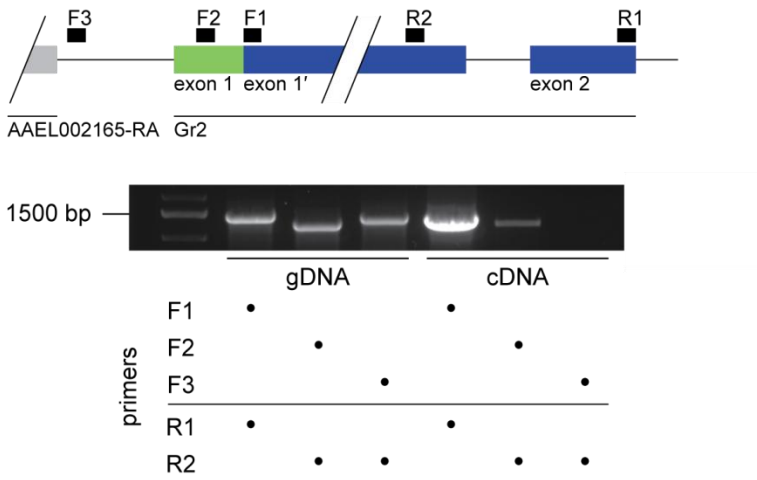


Figure 3.1. Polymorphism in the 5′ region of Gr2. Schematic of the Gr2 gene region with locations of forward and reverse primers (top). The predicted sequence of Gr2 includes a non-conserved 75-bp region at the 5′ end of exon 1 that was not included in the cloned gene. PCR of cDNA shows that while this region is present in mRNA, the (cloned) version without the 5′ extension is substantially more frequent (bottom).

Detailed sequence information for all three cloned genes is available in Appendix 4. Transgenic lines were recombined with

ΔGr21a, *ΔGr63a*, and other existing lines to generate the genotypes detailed in Appendix 5.

The empty neuron system

Recombinant flies were created that express mosquito receptors in ab1C under a Gr63a–GAL4 driver in a *ΔGr21a;ΔGr63a* background (see Appendix 5 for full genotypes).

As expected, ab1C did not respond to CO_2 or any other odorants in *ΔGr21a;ΔGr63a* flies. These neurons also lacked spontaneous activity (Fig. 3.2A).

Sensilla with ab1C expressing all three mosquito GRs responded to CO_2 presented either by puffing 0.3% CO_2 from a pressurized cylinder for a sub-saturation stimulus or by exhaling on the antenna (~4% CO_2 as well as additional odorants) for a saturating stimulus. These sensilla also responded to the strong cpA activators cyclohexanone (Chapter 2), pyridine (Chapter 2), thiazole (Appendix 1), and dimethyl sulfide (D. MacWilliam, personal communication) significantly more than controls. The transgenic ab1C neurons in these sensilla may also have responded to the moderate cpA activators 3-methyl-1-butanol and hexanal (Chapter 2), but the change in total response over empty neuron controls did not reach the level of significance, perhaps because ab1C activity was masked by the activity of ab1A and ab1B (Table 3.1, Fig 3.2).

Expression of AaGr2 and AaGr3 without AaGr1 also resulted in significant responses to cpA activating odors. This combination detects CO_2, whether from a tank or from exhaled breath, equally well as the combination of all three receptors. The combination of these two receptors renders the neuron substantially more sensitive to

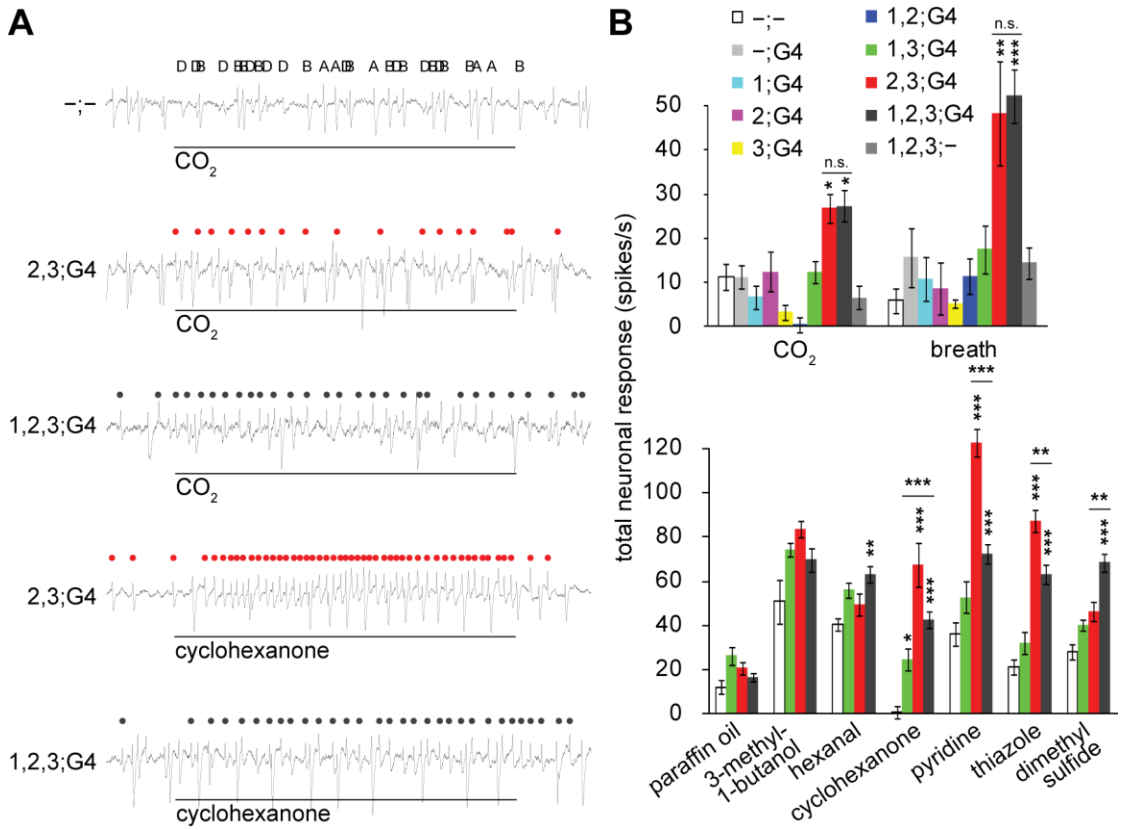


Figure 3.2. Odor responses in the empty neuron system.
(A) Representative traces and (B) mean responses of the ab1 sensillum to the indicated odorants. Letters in (A) indicate action potentials attributed to the ab1A, B, and D neurons; dots indicate action potentials attributed to ab1C. Quantification includes the total responses of all neurons in the sensillum.
n = 8–28, ANOVA followed by Dunnett's test to compare to control genotype −;−. Follow-up ANOVA determined whether responding genotypes differed from each other. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, n.s. not significant. Error bars are s.e.m. See Appendix 6 for full genotypes.

Table 3.1. Odor responses in the empty neuron system.

	baseline	CO_2	breath	PO	i5ol	6al	c6on	prd	thz	dMS	spmd	ibutH
−;−	34.1 ± 3.0 (*n* = 7)	11.2 ± 3.0 (*n* = 9)	5.9 ± 2.8 (*n* = 9)	12.3 ± 3.1 (*n* = 17)	50.8 ± 9.8 (*n* = 8)	40.8 ± 2.8 (*n* = 8)	0.7 ± 2.7 (*n* = 10)	36.3 ± 5.4 (*n* = 10)	21.3 ± 3.4 (*n* = 10)	28.0 ± 3.4 (*n* = 8)	8.1 ± 1.9 (*n* = 10)	12.5 ± 1.9 (*n* = 10)
−;G4	34.9 ± 5.3 (*n* = 7)	11.2 ± 2.6 (*n* = 10)	15.6 ± 6.6 (*n* = 10)	21.3 ± 2.8 (*n* = 14)	65.8 ± 5.5 (*n* = 4)	51.0 ± 4.4 (*n* = 4)	10.1 ± 2.4 (*n* = 10)	34.7 ± 3.1 (*n* = 10)	23.8 ± 2.5 (*n* = 9)	35.2 ± 4.5 (*n* = 4)	16.1 ± 2.8 (*n* = 9)	17.1 ± 1.5 (*n* = 9)
1;G4	30.8 ± 5.8 (*n* = 4)	6.7 ± 2.6 (*n* = 6)	10.8 ± 5.1 (*n* = 6)	13.6 ± 2.8 (*n* = 8)	70.2 ± 9.2 (*n* = 4)	63.8 ± 6.7 (*n* = 4)	5.5 ± 3.0 (*n* = 6)	31.3 ± 2.8 (*n* = 6)	23.0 ± 3.0 (*n* = 5)	25.2 ± 4.3 (*n* = 4)	6.7 ± 2.1 (*n* = 6)	15.3 ± 3.7 (*n* = 6)
2;G4	29.2 ± 2.7 (*n* = 4)	12.5 ± 4.5 (*n* = 6)	8.7 ± 5.9 (*n* = 6)	18.9 ± 2.7 (*n* = 10)	57.8 ± 8.7 (*n* = 4)	46.3 ± 4.8 (*n* = 4)	9.8 ± 2.6 (*n* = 6)	42.8 ± 4.2 (*n* = 6)	25.8 ± 3.9 (*n* = 5)	32.5 ± 6.7 (*n* = 4)	11.7 ± 2.5 (*n* = 6)	10.0 ± 1.2 (*n* = 6)
3;G4	32.0 ± 5.7 (*n* = 4)	3.2 ± 1.7 (*n* = 4)	5.2 ± 0.9 (*n* = 5)	15.6 ± 2.0 (*n* = 8)	ND	ND	−2.8 ± 3.5 (*n* = 4)	29.2 ± 2.1 (*n* = 4)	22.2 ± 7.4 (*n* = 4)	36.5 ± 2.5 (*n* = 4)	10.8 ± 5.2 (*n* = 4)	13.8 ± 4.3 (*n* = 4)
1,2;G4	22.4 ± 3.4 (*n* = 5)	0.5 ± 1.7 (*n* = 6)	11.3 ± 4.0 (*n* = 6)	15.9 ± 3.4 (*n* = 10)	64.0 ± 9.0 (*n* = 4)	49.3 ± 2.0 (*n* = 4)	2.8 ± 4.3 (*n* = 5)	31.0 ± 11.9 (*n* = 6)	21.5 ± 8.8 (*n* = 6)	40.5 ± 4.1 (*n* = 4)	9.3 ± 4.7 (*n* = 6)	17.0 ± 2.8 (*n* = 6)
1,3;G4	25.7 ± 2.2 (*n* = 6)	12.4 ± 2.5 (*n* = 8)	17.5 ± 5.4 (*n* = 8)	26.4 ± 4.1 (*n* = 12)	74.2 ± 2.9 (*n* = 8)	56.0 ± 3.6 (*n* = 8)	24.8 ± 5.2 (*n* = 8)	52.8 ± 7.3 (*n* = 8)	32.2 ± 4.8 (*n* = 8)	40.2 ± 2.7 (*n* = 8)	16.3 ± 2.9 (*n* = 6)	21.8 ± 4.8 (*n* = 8)
2,3;G4	25.4 ± 2.5 (*n* = 10)	26.8 ± 3.3 (*n* = 12)	48.2 ± 11.8 (*n* = 12)	20.8 ± 2.7 (*n* = 20)	83.5 ± 3.9 (*n* = 8)	49.5 ± 4.8 (*n* = 8)	67.4 ± 10.1 (*n* = 12)	122.7 ± 6.2 (*n* = 12)	87.3 ± 5.2 (*n* = 9)	46.5 ± 4.2 (*n* = 8)	6.1 ± 2.7 (*n* = 10)	10.7 ± 2.7 (*n* = 10)
1,2,3;G4	36.0 ± 2.7 (*n* = 25)	27.3 ± 3.6 (*n* = 28)	52.1 ± 6.0 (*n* = 28)	16.9 ± 1.9 (*n* = 50)	69.6 ± 5.2 (*n* = 22)	62.9 ± 3.8 (*n* = 22)	42.9 ± 3.7 (*n* = 27)	72.3 ± 4.2 (*n* = 27)	63.1 ± 4.6 (*n* = 27)	68.5 ± 4.2 (*n* = 22)	5.1 ± 1.3 (*n* = 27)	9.0 ± 1.9 (*n* = 27)
1,2,3;−	23.3 ± 2.3 (*n* = 15)	6.5 ± 2.6 (*n* = 15)	14.4 ± 3.5 (*n* = 15)	17.6 ± 2.1 (*n* = 25)	72.3 ± 5.2 (*n* = 12)	61.8 ± 4.9 (*n* = 12)	12.9 ± 4.6 (*n* = 13)	32.5 ± 6.6 (*n* = 13)	27.5 ± 5.6 (*n* = 13)	43.0 ± 2.5 (*n* = 12)	10.5 ± 1.6 (*n* = 13)	18.0 ± 2.4 (*n* = 13)

Table 3.1. Odor responses in the empty neuron system.
Mean ± s.e.m. (and *n* replicates) of total odor-evoked responses in spikes/s of the neurons in ab1 sensilla of flies with the indicated genotypes, where mosquito gustatory receptors were expressed in ab1C neurons that lacked endogenous receptors. Two outliers in which ab1A atypically responded strongly to odor were removed from analysis for 1,3;G4 responses to spermidine. Responses significantly different from the −;− control genotype, as determined by ANOVA followed by Dunnett's *post-hoc* test, are outlined in red.
baseline: activity before exposure to odors. **PO:** paraffin oil. **i5ol:** 3-methyl-1-butanol. **6al:** hexanal. **c6on:** cyclohexanone. **prd:** pyridine. **thz:** thiazole. **dMS:** dimethyl sulfide. **spmd:** spermidine. **ibutH:** isobutyric acid. **ND:** no data. See Appendix 5 for full genotypes.

other activating odorants than the combination of all three (Fig. 3.2), indicating that co-expression of AaGr1 interferes with or inhibits the response to these odors, but not to CO_2.

Curiously, the combination of AaGr1 and AaGr3 did not respond to CO_2 and also did not respond to cpA activators except cyclohexanone. There was no spontaneous activity attributable to ab1C in these sensilla, suggesting that AaGr1 + AaGr3 does not form a functional receptor. This was unexpected because DmGr21a and DmGr63a, the fly receptors normally expressed in ab1C, are closest in sequence to AaGr1 and AaGr3 (Robertson and Kent 2009).

No single GR expressed by itself conferred odor responses, nor did the combination of AaGr1 and AaGr2 without AaGr3 (Table 3.1).

Fine tuning the ab1C empty neuron system

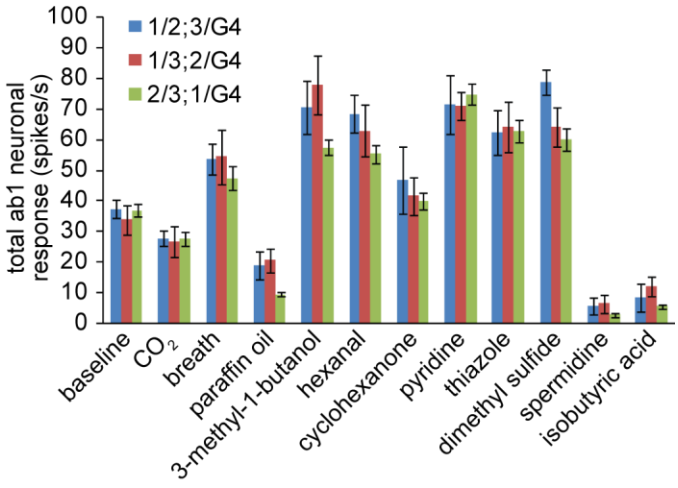


Figure 3.3. Mean odor responses for three genotypes expressing all three mosquito constructs in different arrangements. The effect of genotype was not significant across the dataset, n = 7–10 per odor–genotype combination. ANOVA $p > 0.05$.

Three different genotypes of fly were used to express all three mosquito receptors in ab1C to check for variation due to the position of the gene construct. No substantial differences were observed, so data from these genotypes are pooled in other analyses (Fig 3.3).

One drawback of the ab1C empty neuron system is that many of the odors detected by the mosquito cpA neuron are also sensitively detected by the ab1A and/or ab1B, two of the neurons housed in the same sensillum as ab1C. The recorded spikes corresponding to the action potentials of the four neurons in the ab1 sensillum can be distinguished by their characteristic relative amplitudes. The ab1C neuron has the third largest amplitude, so when either ab1A or ab1B responds strongly to an odor, the activity of ab1C is completely masked. Because of this problem, a subset of cpA activators that do not or only weakly activate ab1A or ab1B were used in these experiments. Still, ab1C responses to odors that evoke more ab1A/ab1B activity such as 3-methyl-1-butanol, hexanal, and dimethyl sulfide are likely underestimated.

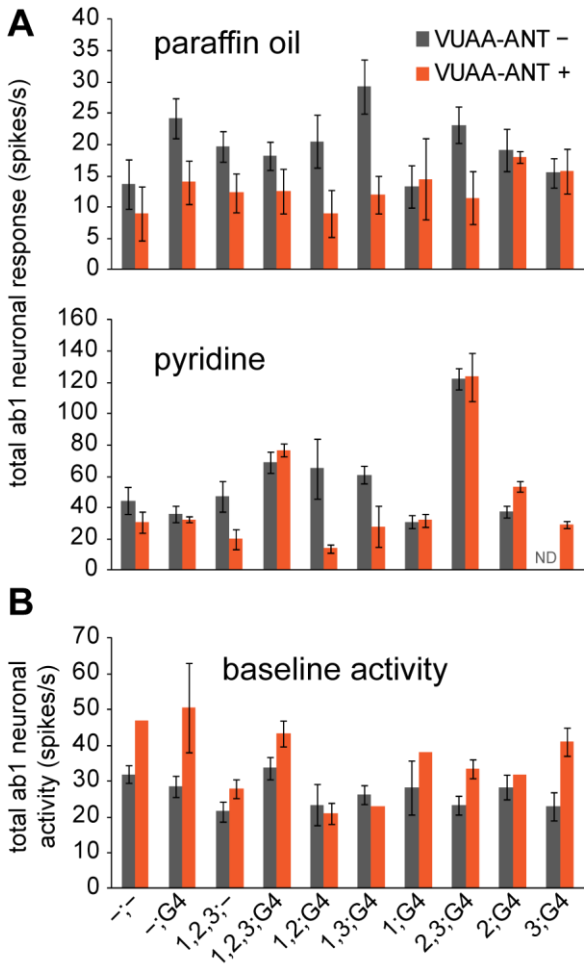


Figure 3.4. Effect of VUAA-ANT on the empty neuron system.
(A) Mean odor-evoked responses in the ab1 sensillum to solvent (top) and to pyridine, which strongly activates ab1C in flies of genotypes 2,3;G4 and 1,2,3;G4.
(B) Mean total neuronal activity prior to stimulation with odorants.
Error bars are s.e.m. **ND:** no data.

An additional issue is that it is not possible to attribute each observed action potential to a particular neuron when more than one is responding simultaneously. Therefore in most cases total neuronal

activity was counted and the activity of test genotypes was compared with the activity of the *ΔGr21a;ΔGr63a* ab1 sensillum, i.e., the total responses attributable to ab1A, ab1B and ab1D.

The pharmaceutical VU0183254, also known as VUAA-ANT, is a specific antagonist of orco (Jones et al. 2011), so it is expected to reduce the responsiveness of ab1A, ab1B, and ab1D to odorants without affecting ab1C activity. Indeed, exposure to VUAA-ANT attenuated the total neuronal response to the solvent paraffin oil across all genotypes, while responses to a strong ab1C activator like pyridine were largely unaffected in responding genotypes (Fig. 3.4A). However, the observed reduction in odor-evoked responses was largely due to an increase in spontaneous firing rate without a corresponding increase in odor-evoked activity (Fig. 3.4B). Thus although the effect of VUAA-ANT was highly significant across the dataset, it was not effective at improving resolution of ab1C activity and its use was discontinued during the experiment. The effect of VUAA-ANT was small, so data from sensilla treated with VUAA-ANT or not are pooled here, but the difference was taken into account for statistical analysis.

Inhibition of the transgenic ab1C neuron

Spermidine and isobutyric acid are both inhibitors of the cpA neuron (Chapter 2; D. MacWilliam, personal communication). In both flies and mosquitoes, spermidine inhibits baseline activity of the CO_2-sensitive neuron. However, it only weakly inhibits mosquito cpA's response to a CO_2 stimulus (D. MacWilliam, personal communication). I found that spermidine did not significantly inhibit ab1C's response to 0.3% CO_2 in wild type sensilla expressing the *Drosophila* receptors DmGr21a and DmGr63a. The response

to the same concentration of CO_2 was slightly inhibited in the transgenic neuron expressing mosquito receptors, but only in the absence of AaGr1.

Isobutyric acid is a component of sweat odor that inhibits cpA's responses to both CO_2 and other activators. It does not inhibit the CO_2 response in *Drosophila* wild type neurons, but does so in both types of transgenic responders (Fig. 3.5). Unlike spermidine, isobutyric acid is a more effective inhibitor when AaGr1 is present.

Co-expressed mosquito and *Drosophila* receptors interact

Mosquito receptors were expressed in ab1C in addition to the endogenous receptors DmGr21a and DmGr63a, that is, in a "full" neuron. Expressing any AaGr or combination of AaGrs shifted the odor tuning profile of the sensillum (Table 3.2). Different AaGr combinations increased or decreased sensillar responses to different odorants in a highly variable manner.

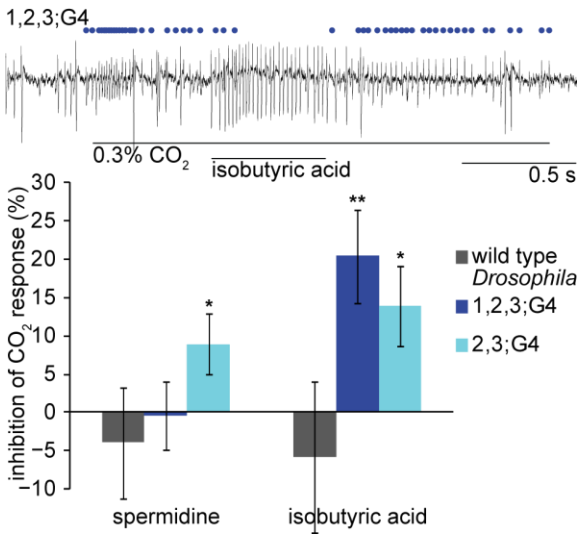


Figure 3.5. Inhibition of CO_2 responses by spermidine and isobutyric acid. Sample trace (top) and mean percent inhibition (bottom) of ab1 sensillar activity during CO_2 stimulation by cpA inhibitors in the indicated genotypes. Isobutyric acid activates ab1A, but inhibits ab1C; dots indicate action potentials of ab1C. Inhibition is calculated from total neuronal activity, so is an underestimate of inhibition of ab1C. n = 7–8. One-sample t test, *p < 0.05, **p < 0.01. Error bars are s.e.m.

To clarify how the receptor subunits interact across species, hybrid receptors were created by expressing receptors from non-*Drosophila* species in flies missing the orthologous (or paralogous) gene. In addition to the *Ae.*

Table 3.2. Odor responses in a full neuron system.

	baseline	CO_2	breath	PO	i5ol	6al	c6on	prd	thz	dMS	spmd	ibutH
wild type	27.6 ± 3.8 (*n* = 7)	43.9 ± 7.1 (*n* = 12)	71.3 ± 9.2 (*n* = 12)	30.4 ± 3.5 (*n* = 14)	82.2 ± 9.1 (*n* = 6)	68.0 ± 4.8 (*n* = 6)	65.8 ± 4.7 (*n* = 12)	96.9 ± 5.5 (*n* = 12)	87.2 ± 6.9 (*n* = 12)	69.3 ± 8.1 (*n* = 6)	22.8 ± 5.0 (*n* = 12)	23.9 ± 3.2 (*n* = 12)
1;G4	19.7 ± 2.9 (*n* = 6)	51.7 ± 7.6 (*n* = 9)	110.2 ± 10.3 (*n* = 4)	26.2 ± 4.5 (*n* = 9)	92.6 ± 3.2 (*n* = 9)	75.4 ± 5.5 (*n* = 9)	48.2 ± 3.7 (*n* = 9)	77.2 ± 8.6 (*n* = 9)	78.9 ± 6.5 (*n* = 9)	63.0 ± 10.6 (*n* = 4)	32.4 ± 2.3 (*n* = 9)	30.0 ± 2.6 (*n* = 9)
2;G4	33.0 (*n* = 1)	30.5 ± 12.5 (*n* = 2)	48.0 (*n* = 1)	16.0 ± 7.0 (*n* = 2)	71.5 ± 20.5 (*n* = 2)	57.5 ± 16.5 (*n* = 2)	29.0 ± 6.0 (*n* = 2)	83.5 ± 21.5 (*n* = 2)	60.0 ± 4.0 (*n* = 2)	ND	4.0 ± 11.0 (*n* = 2)	10.0 ± 10.0 (*n* = 2)
3;G4	27.6 ± 2.4 (*n* = 8)	44.7 ± 5.4 (*n* = 9)	77.8 ± 8.1 (*n* = 8)	27.9 ± 3.2 (*n* = 15)	63.1 ± 5.9 (*n* = 9)	56.3 ± 4.5 (*n* = 9)	45.6 ± 6.5 (*n* = 9)	67.3 ± 7.2 (*n* = 9)	83.9 ± 9.6 (*n* = 9)	68.5 ± 4.0 (*n* = 8)	7.1 ± 3.5 (*n* = 9)	24.6 ± 2.7 (*n* = 9)
1,2;G4	18.5 ± 2.6 (*n* = 4)	39.8 ± 6.9 (*n* = 6)	50.0 ± 11.5 (*n* = 3)	20.6 ± 2.9 (*n* = 7)	69.1 ± 9.2 (*n* = 7)	77.7 ± 4.6 (*n* = 7)	46.0 ± 7.0 (*n* = 7)	78.0 ± 9.4 (*n* = 7)	58.6 ± 5.9 (*n* = 7)	65.5 ± 11.0 (*n* = 4)	13.7 ± 3.9 (*n* = 7)	22.1 ± 3.2 (*n* = 7)
1,3;G4	34.0 ± 7.7 (*n* = 4)	53.0 ± 4.6 (*n* = 8)	77.5 ± 12.4 (*n* = 6)	20.8 ± 6.3 (*n* = 8)	89.8 ± 8.5 (*n* = 5)	72.0 ± 5.3 (*n* = 5)	30.6 ± 4.5 (*n* = 7)	56.6 ± 9.6 (*n* = 7)	61.4 ± 9.2 (*n* = 7)	63.8 ± 3.3 (*n* = 4)	2.0 ± 2.8 (*n* = 7)	20.7 ± 4.1 (*n* = 7)
2,3;G4	26.2 ± 1.0 (*n* = 4)	44.5 ± 4.3 (*n* = 8)	81.1 ± 9.4 (*n* = 8)	33.1 ± 4.0 (*n* = 8)	98.1 ± 6.7 (*n* = 8)	72.5 ± 4.9 (*n* = 8)	72.8 ± 5.9 (*n* = 8)	116.8 ± 10.9 (*n* = 8)	111.8 ± 9.8 (*n* = 8)	67.5 ± 4.7 (*n* = 8)	21.4 ± 2.5 (*n* = 8)	27.5 ± 2.5 (*n* = 8)
1,2,3;G4	34.7 ± 4.0 (*n* = 10)	31.3 ± 4.0 (*n* = 19)	42.5 ± 4.6 (*n* = 17)	19.3 ± 2.4 (*n* = 19)	67.8 ± 11.9 (*n* = 8)	57.4 ± 10.0 (*n* = 8)	41.2 ± 4.1 (*n* = 19)	79.5 ± 8.0 (*n* = 19)	70.0 ± 6.6 (*n* = 18)	62.2 ± 19.4 (*n* = 4)	13.2 ± 2.2 (*n* = 18)	15.2 ± 1.8 (*n* = 18)
1,2,3;+	34.4 ± 4.7 (*n* = 5)	42.2 ± 5.1 (*n* = 10)	66.8 ± 14.4 (*n* = 8)	23.2 ± 4.4 (*n* = 10)	52.8 ± 6.4 (*n* = 5)	46.8 ± 5.0 (*n* = 5)	40.0 ± 6.1 (*n* = 9)	58.7 ± 8.8 (*n* = 9)	66.9 ± 13.4 (*n* = 9)	55.5 ± 11.5 (*n* = 2)	17.8 ± 5.6 (*n* = 9)	22.1 ± 5.4 (*n* = 9)

Table 3.2. Odor responses in a full neuron system.
Mean and s.e.m. of total odor-evoked responses in spikes/s of the neurons in ab1 sensilla of flies with the indicated genotypes, where mosquito gustatory receptors were expressed in ab1C neurons along with both endogenous receptors. Responses significantly different from the wild type, as determined by ANOVA followed by Dunnett's *post-hoc* test, are outlined in red. **baseline:** activity before exposure to odors. **PO:** paraffin oil. **i5ol:** 3-methyl-1-butanol. **6al:** hexanal. **c6on:** cyclohexanone. **prd:** pyridine. **thz:** thiazole. **dMS:** dimethyl sulfide. **spmd:** spermidine. **ibutH:** isobutyric acid. **ND:** no data. See Appendix 5 for full genotypes.

Table 3.3. Odor responses with hybrid receptors.

	baseline	CO_2	breath	PO	i5ol	6al	c6on	prd	thz	dMS	spmd	ibutH
−;−	34.1 ± 3.0 (*n* = 3)	11.2 ± 3.0 (*n* = 4)	5.9 ± 2.8 (*n* = 4)	12.3 ± 3.1 (*n* = 6)	50.8 ± 9.8 (*n* = 2)	40.8 ± 2.8 (*n* = 2)	0.7 ± 2.7 (*n* = 4)	36.3 ± 5.4 (*n* = 4)	21.3 ± 3.4 (*n* = 4)	28.0 ± 3.4 (*n* = 2)	8.1 ± 1.9 (*n* = 4)	12.5 ± 1.9 (*n* = 4)
Δ21a,Aa1;G4	21.8 ± 2.2 (*n* = 5)	12.6 ± 4.1 (*n* = 9)	28.2 ± 6.8 (*n* = 9)	21.2 ± 3.0 (*n* = 9)	86.0 ± 10.0 (*n* = 9)	86.2 ± 9.9 (*n* = 9)	18.1 ± 6.0 (*n* = 9)	46.1 ± 8.5 (*n* = 9)	38.0 ± 6.1 (*n* = 9)	48.8 ± 5.9 (*n* = 8)	11.3 ± 2.8 (*n* = 9)	17.8 ± 2.7 (*n* = 9)
Δ21a,Aa2;G4	23.5 ± 7.1 (*n* = 4)	11.8 ± 2.4 (*n* = 8)	27.5 ± 5.1 (*n* = 8)	27.1 ± 3.2 (*n* = 8)	67.0 ± 5.3 (*n* = 8)	61.2 ± 4.3 (*n* = 8)	44.0 ± 7.9 (*n* = 8)	77.4 ± 9.6 (*n* = 8)	52.5 ± 7.8 (*n* = 8)	48.9 ± 3.6 (*n* = 8)	19.4 ± 5.6 (*n* = 8)	24.2 ± 3.5 (*n* = 8)
Δ21a,Aa1/2;G4	24.0 ± 2.4 (*n* = 4)	22.2 ± 5.3 (*n* = 8)	49.5 ± 8.4 (*n* = 8)	33.5 ± 5.7 (*n* = 8)	89.5 ± 9.1 (*n* = 8)	68.6 ± 6.2 (*n* = 8)	85.0 ± 4.6 (*n* = 8)	85.6 ± 16.8 (*n* = 8)	90.2 ± 8.1 (*n* = 8)	64.2 ± 5.6 (*n* = 8)	25.0 ± 8.4 (*n* = 8)	38.4 ± 6.5 (*n* = 8)
Aa3;Δ63a,G4	30.2 ± 4.8 (*n* = 3)	8.0 ± 2.7 (*n* = 5)	11.3 ± 6.2 (*n* = 5)	6.3 ± 4.5 (*n* = 5)	83.8 ± 12.2 (*n* = 4)	66.5 ± 3.9 (*n* = 4)	20.7 ± 8.6 (*n* = 5)	17.9 ± 3.3 (*n* = 5)	13.0 ± 3.2 (*n* = 5)	39.8 ± 3.4 (*n* = 4)	16.3 ± 3.1 (*n* = 5)	15.4 ± 2.9 (*n* = 5)
Δ21a,Ag22;G4	14.0 ± 4.0 (*n* = 2)	4.2 ± 3.2 (*n* = 4)	20.8 ± 10.2 (*n* = 4)	19.2 ± 4.2 (*n* = 4)	65.5 ± 1.8 (*n* = 4)	75.0 ± 6.0 (*n* = 4)	18.8 ± 6.6 (*n* = 4)	46.8 ± 12.2 (*n* = 4)	33.8 ± 7.3 (*n* = 4)	36.7 ± 2.4 (*n* = 3)	19.2 ± 8.0 (*n* = 4)	37.8 ± 2.1 (*n* = 4)
Δ21a,Ag23;G4	28.5 ± 4.2 (*n* = 4)	13.8 ± 2.4 (*n* = 9)	30.6 ± 4.3 (*n* = 9)	32.7 ± 1.6 (*n* = 9)	71.6 ± 5.9 (*n* = 8)	62.0 ± 4.3 (*n* = 8)	44.9 ± 5.2 (*n* = 8)	69.9 ± 6.9 (*n* = 8)	47.6 ± 4.1 (*n* = 8)	45.0 ± 2.5 (*n* = 8)	24.9 ± 3.8 (*n* = 8)	29.2 ± 2.3 (*n* = 8)
Δ21a,Ag22/23;G4	38.5 ± 12.5 (*n* = 2)	21.2 ± 2.8 (*n* = 4)	55.8 ± 13.5 (*n* = 4)	14.0 ± 2.0 (*n* = 4)	65.2 ± 5.1 (*n* = 4)	45.0 ± 4.9 (*n* = 4)	35.2 ± 5.0 (*n* = 4)	84.0 ± 8.3 (*n* = 4)	56.2 ± 5.8 (*n* = 4)	48.5 ± 9.4 (*n* = 4)	2.2 ± 4.5 (*n* = 4)	19.5 ± 4.4 (*n* = 4)
Ag24;Δ63a,G4	25.0 ± 6.0 (*n* = 2)	2.8 ± 4.9 (*n* = 4)	0.8 ± 8.6 (*n* = 4)	16.8 ± 4.3 (*n* = 4)	65.0 ± 11.2 (*n* = 4)	56.8 ± 7.5 (*n* = 4)	40.0 ± 14.3 (*n* = 4)	42.8 ± 2.7 (*n* = 4)	39.8 ± 11.8 (*n* = 4)	35.2 ± 5.7 (*n* = 4)	4.8 ± 3.3 (*n* = 4)	19.0 ± 3.2 (*n* = 4)
Δ21a,Pp22;G4	21.7 ± 3.4 (*n* = 3)	3.5 ± 5.0 (*n* = 4)	−1.5 ± 3.6 (*n* = 4)	14.6 ± 4.4 (*n* = 5)	60.2 ± 3.4 (*n* = 4)	75.2 ± 10.7 (*n* = 4)	12.2 ± 5.2 (*n* = 4)	34.8 ± 3.4 (*n* = 4)	28.5 ± 3.8 (*n* = 4)	43.8 ± 1.5 (*n* = 4)	5.5 ± 4.3 (*n* = 4)	26.0 ± 5.6 (*n* = 4)
Pp24;Δ63a,G4	17.0 ± 2.0 (*n* = 2)	2.8 ± 3.8 (*n* = 4)	9.5 ± 2.5 (*n* = 4)	16.0 ± 5.0 (*n* = 6)	67.5 ± 8.5 (*n* = 4)	62.0 ± 6.9 (*n* = 4)	36.5 ± 12.8 (*n* = 4)	39.8 ± 8.2 (*n* = 4)	41.5 ± 7.1 (*n* = 4)	39.0 ± 6.3 (*n* = 4)	11.8 ± 2.7 (*n* = 4)	25.5 ± 5.1 (*n* = 4)

Table 3.3. Odor responses with hybrid receptors.
Mean and s.e.m. of total odor-evoked responses of the neurons in ab1 sensilla of flies with the indicated genotypes, where mosquito gustatory receptors were expressed in ab1C neurons missing the *Drosophila* ortholog or paralog. Responses significantly different from the −;− control genotype, as determined by ANOVA followed by Dunnett's *post-hoc* test, are outlined in red.
baseline: activity before exposure to odors. **PO:** paraffin oil. **i5ol:** 3-methyl-1-butanol. **6al:** hexanal. **c6on:** cyclohexanone. **prd:** pyridine. **thz:** thiazole. **dMS:** dimethyl sulfide. **spmd:** spermidine. **ibutH:** isobutyric acid. See Appendix 5 for full genotypes.

aegypti GRs, receptors from the malaria mosquito *An. gambiae*, and the sand fly *Phlebotomus papatasi* (Scopoli, 1786) were also tested in this manner.

DmGr21a did not function with the Gr63a orthologs from any other of the tested dipteran species (AaGr3, Ag24, or Pp24), except for AgGr24: that receptor had a weak response to cyclohexanone only. Sensillar responses to all tested odorants were indistinguishable from the empty neuron (Table 3.3), and ab1C had no apparent spontaneous activity.

DmGr63a, however, did confer odor responses when expressed in combination with paralogs of DmGr21a, including Gr1 homologs, Gr2 homologs, or combinations of both (Table 3.3). When both AaGr1 and AaGr2 were expressed in a cell with endogenous DmGr63a, they conferred responses to CO_2 and a range of other odorants. A similar trend was observed with the homologous *An. gambiae* genes AgGr22 and AgGr23, though only the response to exhaled CO_2 reached the level of significance (Fig. 3.6, Table 3.3). When either Gr1 or Gr2 was expressed alone with endogenous DmGr63a, they did not confer a CO_2

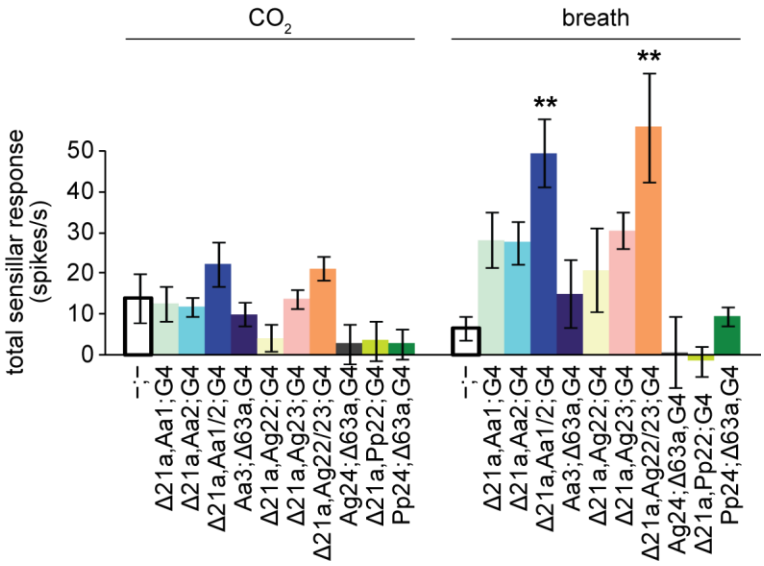


Figure 3.6. CO_2 responses with hybrid receptors. Mean responses of ab1 sensilla with the indicated genotypes to 0.3% CO_2 or exhaled breath (~4% CO_2). n = 4–9, ANOVA followed by Dunnett's test to compare to control genotype −;−. $^{**}p < 0.01$. Error bars are s.e.m. See Appendix 6 for full genotypes.

response, but Gr1 conferred a hexanal response and Gr2 conferred a cyclohexanone response (Table 3.3).

Discussion

In every case where any member of the Gr1,2,3 clade has been found in a particular insect species, all three are clearly present, with the sole exception of the genus *Drosophila* (e.g., Robertson and Kent 2009). Since the *Drosophila* homologs of Gr1 and Gr3 make a functional receptor, I originally hypothesized that the mosquito receptors AaGr1 and AaGr3 would likewise work together to confer responses to CO_2 and other odorants. However, the combination of AaGr1 and Gr3 did not yield responses to any odorants except for a weak response to cyclohexanone, while the combination of AaGr2 and Gr3 responded to many odorants. The UAS–AaGr1 construct used in these experiments was inserted into the same location on the genome as UAS–Gr2 and did affect odor responses in other contexts, so there is no reason to suspect that the construct was simply nonfunctional. DmGr21a is much closer in primary sequence to AaGr1 (65% amino acid identity) than to AaGr2 (36%), and there is no region of sequence that is shared between DmGr21a and AaGr2 that is not also shared with AaGr1 (Fig. 3.6A).

Drosophila receptors can interact with homologous receptors from other species, but this happens asymmetrically: in most cases, DmGr21a did not combine with any tested Gr3 homolog well enough to confer odor sensitivity, but DmGr63a did. In this respect, DmGr21a actually resembles its Gr1 orthologs, since they also do not confer an odor response when co-expressed with Gr3. It is possible that the observed functional difference between DmGr21a and AaGr1 is not due to major structural differences


A
AaGr1 MIHSQMEDSQYQIRQQILNPNQRQQLEDNRRIKEQMQQLQRDDASPSHMYIRKLEFQADVNLLDKHDSFYHTTKSLLVLFQIMGVMPIVRSPKGVNMPRTTFT
DmGr21a MSFWAVSRGLTPPSKVVPMLNPNQRQFLEDEVRYREKLKLMARGDAM-EEVYVRKQETVDDPLELDKHDSFYQTTKSLLVLFQIMGVMPIHRNPPEKNLPRTGYS
AaGr2 MVIKDSEFEDSLNYALLRGDMGTTWDINKDERMMNGTLDPELIQRAKERAIRAQLNSADGDTCELHDQFYRDHKLLLVLFRALAVMPILRSSPG----RITFD
AaGr1 WCSKAFIWAYFIYACETVLVVLVAKERIKRFISTSDKRFDEVIYNIIFMSLLVPHFLLPVASWRNGSEVAKFKNMWTDYQYKYLMVTGKPIVFPKLYPITWVLCV
DmGr21a WGSKQVMWAIFIYSCQTTIVVLVLRERVKKFVTSPDKRFDEAIYNVIFISLLFTNFLLPVASWRHGPQVAIFKNMWTNYQYKFFKTTGSPIVFPNLYPLTWSLCV
AaGr2 WRSWASIYAYCFYVVSTVIVLIVGYERLK--ILQDTKKFDEYIYGVLFIIFLVPHFWIPFVGWGVAKHVAVYKTMWGAFQVRYYRVTGTNLQFPHLKILIVMFSI
AaGr1 VSWAVSFVIIMSQYYLQPDFQLTHTFAYYHILAMLNGFCSLWFVNCTAFGTASKAFAQELSNIFATEQPADKLTEYRHLWVDLSHMMQQLGKAYSNMYGIYCLVI
DmGr21a FSWLLSIAINLSQYFLQPDFRLWYTFAYYPIIAMLNCFCSLWYINCNAFGTASRALSDALQTTIRGEKPAQKLTEYRHLWVDLSHMMQQLGRAYSNMYGMYCLVI
AaGr2 GCLVCAIVFLLSLSFLLEGFALWHTSAYYHIITMLNMNSALWYINCRGIRVASSSLSDRFRKDVAIECTAAMISQYRFLWLNLSELLQALGNAYARTYSTYCLFM
AaGr1 FFTTIIATYGALSEIIEHGA--TYKEVGLFVIVFYCMGLLFIICNEAHHASRRVGLNFQERLLNVNLTAVDKATQKEVEMFLVAIDKNPPTMNLDGYANINRGLI
DmGr21a FFTTIIATYGSISEIIDHGA--TYKEVGLFVIVFYCMGLLYIICNEAHYASRKVGLDFQTKLLNINLTAVDAATQKEVEMLLVAINKNPPIMNLDGYANINRELI
AaGr2 FANITIAIYGALSEVIDHGFGFSFKEIGLIVDTVYCSTLLFIFCDCSHNATLQVAQGVQDTLLGINLLKVDHPTQKEIDLFIQAIEMNPAIVSLKGYAEVNRELL
AaGr1 TSNISFMATYLVVLMQFKLTLLRQSARKALIPALRANLTKLKEN-
DmGr21a TTNISFMATYLVVLLQFKITEQRRIGQQQA-
AaGr2 TASIATIAIYLVVLLQFKLSLISQQMPIELMEIKHSHKG-
B
AaGr3 MNLNQDPIQYINLNNNARTVFLDVKPIYNEEKRKVSNGFNNRIGF----PPISSRRVFGLESDFNT--RSDIVYGTTKPIYNVLRMLGVFPFSRPSPGVTLFACA
DmGr63a MANYYRRKKGDAVFLNAKPLNSANAQAYLYGVRKYSIGLAERLDADYEAPPLDRKKSSDSTASNNPEFKPSVFYRNIDPINWFLRIIGVLPIVRHGPARAKFEMN
AaGr3 SPAMAYCSVFFVTLMAYVIYITILRVHIVRTLEGRFEEAVIAYLFIVNILPVLIIPLMWYETRKVSSLLNQWVDFEAIYRKTAGRELELSFRTKALLIAILLPVL
DmGr63a SASFIYSVVFFVLLACYVGYVANNRIHIVRSLSGPFEEAVIAYLFLVNILPIMIIPILWYEARKIAKLFNDWDDFEVLYYQISGHSLPLKLRQKAVYIAIVLPIL
AaGr3 SCLAVIITHVTMVEFQLVQVIPYCILDTLTYMMGGYWYMTCETLSITANILAEDFQRALRHVGPAAMVSEYRSLWLRLSKLARETGSSTCYTFTFLCLYLFFIIT
DmGr63a SVLSVVITHVTMSDLNINQVVPYCILDNLTAMLGAWWFLICEAMSITAHLLAERFQKALKHIGPAAMVADYRVLWLRLSKLTRDTGNALCYTFVFMSLYLFFIIT
AaGr3 LSIYGLMSQISEGFGIKDIGLAVTAFCSVGLLFFICDEAHYASFNVRTKFQKKLLMAELSWMNSDAQTEINMFLRATEMNPSSINLGGFFDVNRTLFKSLLATMV
DmGr63a LSIYGLMSQLSEGFGIKDIGLTITALWNIGLLFYICDEAHYASVNVRTNFQKKLLMVELNWMNSDAQTEINMFLRATEMNPSTINCGGFFDVNRTLFKGLLTTMV
AaGr3 TYLVVLLQFQISIPDDSSMLVMHNMTGSYRE
DmGr63a TYLVVLLQFQISIPTDKGDSEGANNITVVDFVMDSLDNDMSLMGASTLSTTTVGTTLPPPIMKLKGRKG
transmembrane domain
* identical residue

Figure 3.7. Sequences of *Aedes aegypti* and *Drosophila melanogaster* receptors. Aligned amino acid sequences of AaGrs and DmGrs, with identical residues indicated. *Ae. aegypti* receptor sequences are as cloned; *D. melanogaster* sequences are from Flybase.

between DmGr21a and AaGr1, but rather because DmGr63a has changed, perhaps by adapting to the loss of Gr2 by interacting more closely with DmGr21a to form a functional receptor. Indeed, *Drosophila* Gr63a has diverged more from other insects' Gr3 than Gr21a has from Gr1 (Robertson and Kent 2009) and differs substantially in sequence from AaGr3 at the N terminus (Fig. 3.6B), which may be a site of protein–protein interaction. All of the hybrid receptors tested that had only one of Gr1 or Gr2 had weak responses to a subset of odorants, which may also be explained by altered protein–protein interactions between those receptors and DmGr63a. This could be investigated further by engineering a new Gr63a with the N terminus from AaGr3 and pairing it again with AaGr1, AaGr2, and DmGr21a.

The data from the empty neuron clearly support a model in which Gr2 and Gr3 form a functional receptor whose activity is modulated by the presence of Gr1. Gr1 appears to increase the selectivity of the neuron for CO_2, damping down responses to other activating odorants and preventing spermidine from inhibiting CO_2 responses. Gr1 does not interfere with inhibition of CO_2 by isobutyric acid, but rather facilitates it, suggesting that isobutyric acid, which has a very different structure from spermidine, acts through a different binding site. It will be interesting to investigate how these inhibitors interact with non-CO_2 activators with or without Gr1 present.

Decoding GRs in a neuron that natively expresses GRs has clear advantages, but it has only recently become possible to do this in a genuinely "empty" neuron with the creation of the *ΔDmGr21a* mutant. The ab1C neuron has previously been used to decode sweet taste GRs (Freeman et al. 2014), but it was not possible to rule out interactions

between those GRs and Gr21a. The empty neuron system described here solves that problem and is flexible enough to be used in studies of olfactory GRs from different species of insects, including species of agricultural or medical importance.

Methods

Insects

Drosophila melanogaster. Flies were reared on standard cornmeal–dextrose medium at 25°. Complete genotypes and sources of flies used are listed in Appendix 5.

Aedes aegypti. Receptors were cloned from Orlando strain mosquitoes; this is a laboratory strain that was probably established in Orlando, FL, in 1939 or 1942 (Kuno 2010). Mosquitoes were reared at 27°, 70% relative humidity, and L:D 14:10. Larvae were fed on alfalfa pellets; adults were fed 10% sucrose solution. Colonies were maintained by females bloodfed on bovine blood provided through a membrane feeder.

Molecular biology

ΔGr21a flies were created by Sarah Perry in the Ray laboratory using the CRISPR–Cas9 targeted mutagenesis system. The mutant line used in these experiments has a 5 base pair deletion at position 137 in the first exon, resulting in a complete loss of function (S. Perry, personal communication).

To create cDNA for cloning, 55 sets of mouthparts were collected over liquid nitrogen from 5-day-old female mosquitoes. These mosquitoes were mated but not bloodfed. Tissue was Trizol extracted, treated with DNase, and mRNA was retrotranscribed to cDNA by a SuperScript III reaction (Life Technologies). Cloned

genes were amplified using Q5 proof-reading DNA polymerase and gel purified before use.

AaGr1 and *AaGr3* were cloned using the pENTR/D-TOPO system (Life Technologies). DNA from transformed *E. coli* bearing the entry vector was sequenced to confirm successful insertion of the transgene. This system was not successful at cloning *AaGr2*, so the pATTL system was used for this gene. The pATTL vector, developed by G. M. Pask, is similar to pENTR, but instead of the CACC Kozak sequence at the 5′ end of the insertion site, has both an AscI restriction site at the 5′ end (including a different, modified Kozak sequence) and a PacI restriction site at the 3′ end. Using both of these 8-nucleotide restriction sites ensures directional and specific insertion of a restriction site-flanked transgene. *AaGr2* was successfully inserted into this plasmid to create an entry vector.

All three cloned genes were transferred to the pUASg-attB-DV destination vectors and sent out for injection into *Drosophila* embryos by Genetic Services, Inc. (Sudbury, MA) for site-directed transgenic insertion using the ΦC31 system. Transgenes were inserted into three sites: the attP40 site on chromosome II and the attP2 and VK00027 sites on chromosome III. Individual transgenic flies marked by mini-white were used to generate isogenic lines, and presence of the UAS–AaGr construct was confirmed by PCR.

Recombinant flies with loss-of-function mutations in *DmGr21a* and *DmGr63a* and UAS–AaGr constructs on the same chromosomes were created and PCR-confirmed using standard techniques.

Sanger sequencing was performed by Retrogen, Inc. (San Diego, CA).

Odors and stimulus presentation

Chemicals were obtained at the highest purity commercially available, typically >98% (Sigma-Aldrich), and dissolved in paraffin oil at 10^{-2} (1% volume/volume). Odor cartridges were contructed for electrophysiology by applying 50 µl of dissolved odorant onto the cotton plug of a 5¾" Pasteur pipet capped with a blue tip and sealed with Parafilm between uses. Stimuli were presented in the same order across replicates; each cartridge was used for ≤3 stimuli. A constant 5–7 ml/s stream of carbon filtered room air was switched from a blank cartridge to the odor cartridge using a Syntech CS-55. The resulting airflow was delivered into a glass tube with a constant, humidified airstream (10 ml/s) whose mouth was centered on and ~1 cm from the insect head. CO_2 stimuli were pulsed using a PM8000 microinjector (MicroData Intrument, Inc.) to deliver controlled pulses from pressurized cylinders of 1% CO_2 in air into the same carrier airstream, resulting in the indicated final concentration of gas at the insect head.

VU0183254, or VUAA-ANT, was used as described by Jones et al. (2012) in some recordings to reduce responses of neurons other than ab1C. VUAA-ANT was purchased at 95% purity (Enamine Ltd.) and diluted in dimethyl sulfoxide to 0.1M concentration. It was subsequently diluted in sensillum lymph ringers (Kaissling and Thorson 1980) to 10^{-3} M, and this solution was used in the recording electrode as described below.

Electrophysiology

Single-sensillum recordings. Adult female *Drosophila* (4–7 days old) were used for recordings. Each fly was restrained in a truncated pipet tip and placed on a microscope slide with its antenna propped up and stuck to an elevated cover slip with double-stick tape (3M). The antenna was pressed into the tape on the cover slip with a blunt glass needle to keep it from moving. A reference electrode, consisting of a silver wire electrode inserted into a glass micropipet filled with sensillum lymph ringer, was inserted into the fly's eye. A recording electrode, consisting of a silver wire electrode inserted into a glass micropipet filled with sensillum lymph ringer or VUAA-ANT dissolved in sensillum lymph ringer, was inserted into the shaft of a large basiconic sensillum using a micro-manipulator under magnification.

Signals were amplified 1000× and band-pass filtered to admit signals between 10 Hz–1.0 kHz with a Iso-Dam amplifier (World Precision Instruments). Signals were digitized with a Digidata 1440 (Molecular Devices). Recordings were analyzed in the AxoScope and pClamp programs (Molecular Devices); spikes (i.e., action potentials) were counted manually. Because it is not possible to confidently attribute a spike to a particular neuron when multiple neurons respond to an odor, total spikes from all four neurons were counted. Sensillar identity was confirmed by checking responses to diagnostic odorants ethyl acetate, butanedione, and methyl salicylate (all dissolved at 10^{-4} in paraffin oil) before the panel of test odorants. Sensillar responses to test odorants were corrected for baseline firing rate: reported firing frequencies were calculated as 2 × (number of spikes during 0.5 s stimulus presentation) – (number of spikes during 1 s prior

to stimulus presentation). Percent inhibition was calculated relative to the response to a solvent control in the same sensillum.

Statistics

All statistical analyses were performed in R. Electrophysiological data were recorded with 1–2 replicates per insect. Sensillar responses were analyzed by two-factor ANOVA (with genotype and presence of VUAA-ANT as factors) with Type III sum of squares to compensate for unequal sample sizes due to the use of VUAA-ANT. In cases where VUAA-ANT did not have a significant effect, it was dropped from the model and a one-way ANOVA was performed, as this was a more informative model. In either case, the ANOVA was followed by *post-hoc* Dunnett's test to compare test genotypes to the control genotype.

Chapter 4. Contributions of odorant receptors and olfactory gustatory receptors to discrimination among humans by *Aedes aegypti*

Mosquitoes are more attracted to some people more than others. This preference is presumed to be primarily determined by differences in odor between individuals, but clear experimental evidence for which odors are involved has been elusive. The yellowfever mosquito *Aedes aegypti* (L., 1762) is a model species for host-seeking in laboratory bioassays, and females of this anthropophilic species readily discriminate between sources of human odor from different people (Steib et al. 2001). Previous studies (e.g., Logan et al. 2008) have analyzed odors from "more attractive" or "less attractive" individuals to look for odor components that correlate with preference, but when candidate compounds were added to human odor, they had minimal effects on mosquito behavior at biologically relevant concentrations, although some effects were noted at concentrations orders of magnitude greater than their abundance in skin odor (Logan et al. 2008, Logan et al. 2010). I am taking a different approach by querying the mosquito rather than the odor: if an olfactory receptor or olfactory neuron class can be identified that is required for normal preference behavior, then the volatiles detected by that receptor or receptor class are excellent candidates for determining preference. Many of the behavior experiments described below were carried out with the assistance of or entirely by P. U. Ngo under my supervision. We tested mosquitoes with lack-of-function mutations that render receptors from the odorant receptor (OR) gene family or the CO_2- and skin odor–sensitive gustatory receptors (GRs) nonfunctional. Here we present the

results so far, showing that loss of olfactory receptors fromeither of these gene families alters host preference in some contexts.

Ae. aegypti discriminate between odors of socks worn by different people

To assay mosquito olfactory preference, we hung nylon socks that had been worn by two different people on either side of a cage of hungry mosquitoes (Fig. 4.1). The apparatus was kept isolated behind a transparent partition to avoid introducing odors from the experimenter. Mosquitoes consistently preferred odor from a sock worn by the author over another person's (person 314's) sock across replicates conducted on three separate days over two weeks. Participation was high, with typically between 60–80% of mosquitoes in the cage responding even without an activating CO_2 stimulus. A plateau in observable preference reached after the first 2–3 min. One possible explanation for the observed preference is that some component(s) of the odor from person 314 made the sock less acceptable to the test mosquitoes. This was not the case: when a sock from

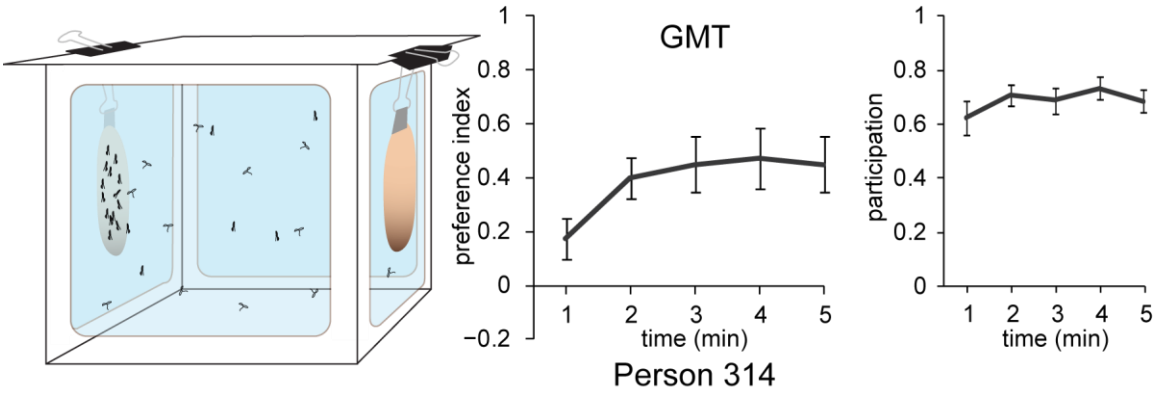


Figure 4.1. A cage-based assay for host preference.
(A) Schematic of the experimental apparatus.
(B) Mosquitoes prefer the author's odor over person 159's. Preference and participation are shown over the course of a 5-min assay. $n = 8$, error bars are s.e.m.

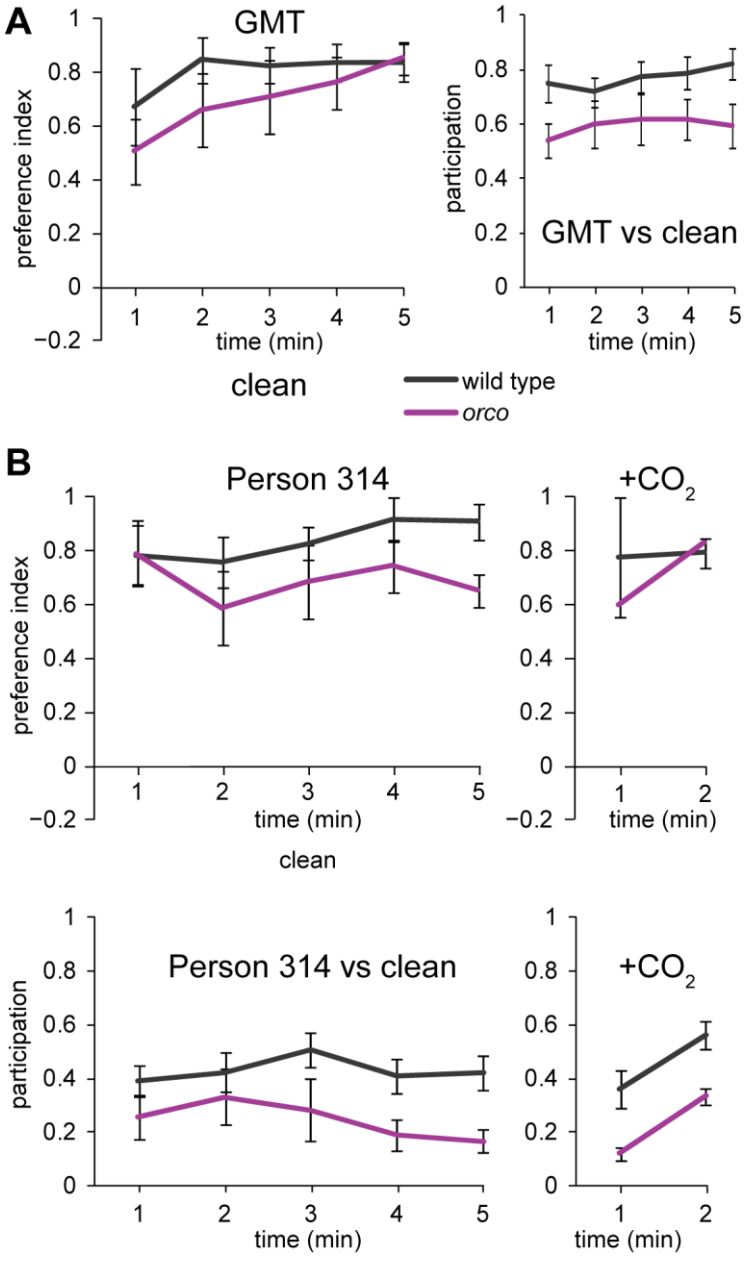


Figure 4.2. One-choice tests. Preference and participation of wild type and *orco* mutant mosquitoes with (A) the author's odor on one side and a clean sock on the other; (B) odor from person 314 on one side and a clean sock on the other, without (left) and with (right) added CO_2.
n = 5–6, error bars are s.e.m.

either GMT or person 314 is tested against a clean sock, mosquitoes show clear and consistent preference for the human odor–laden sock (Fig. 4.2). Thus, both odors were attractive on their own. Participation was slightly lower when 314 was tested against a clean sock, so CO_2 was introduced at the end of the assay to activate mosquitoes. This increased participation but had no effect on observed preference.

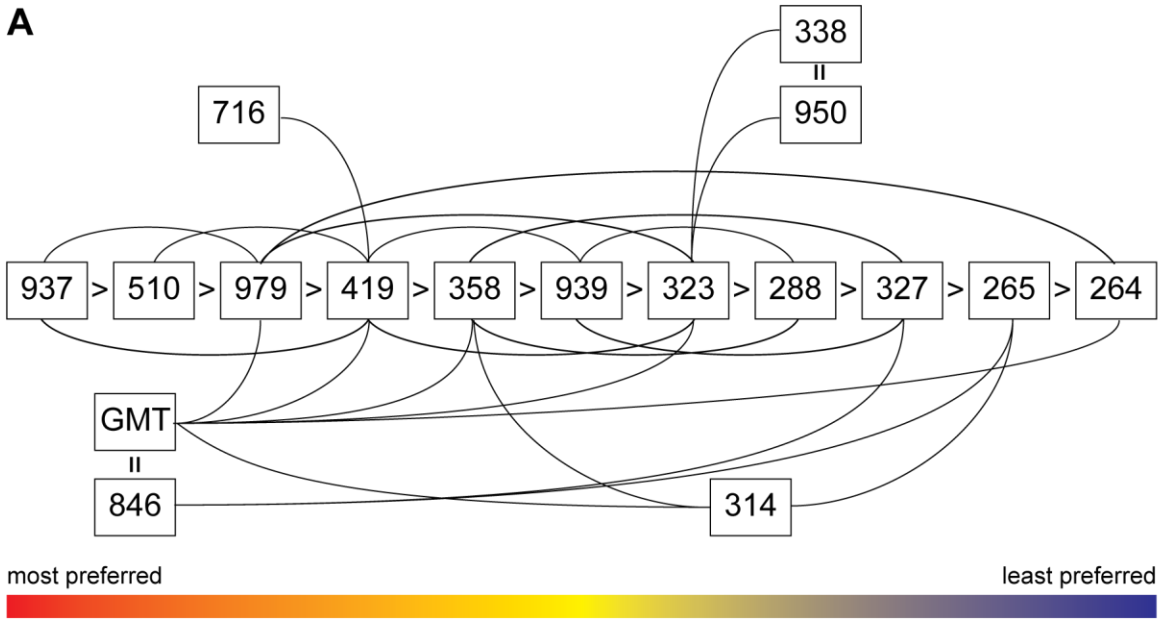
Mutant mosquitoes lacking the co-receptor orco and thus without functional ORs were still strongly attracted to human skin odor with added CO_2 (DeGennaro et al. 2013). Consistent with

this observation, we saw that *orco* mutant mosquitoes preferred an odor-laden sock to a similar degree as wild-type mosquitoes, albeit with slightly lower participation (Fig 4.2).

Attractiveness to mosquitoes falls on a continuum

Additional participants were recruited to the study to investigate the phenomenon of host preference across more than just two individuals. Participants were asked to keep their diet and exercise routines consistent on each day of collection to minimize changes in odor from day to day. These and other factors that could alter odor were deliberately not controlled between subjects to capture more of the natural range of variation in odor. In all cases where it was tested, odor preference was found to be transitive: that is, if odor from person A was preferred over person B and person B was preferred over person C, then person A was preferred over person C. Thus individuals could be ranked in attractiveness to mosquitoes on a linear spectrum. In a series of pairwise comparisons, 11 subjects were ranked in odor preference; six other participants withdrew during the study and were not localized completely within the spectrum (Fig 4.3A). Most comparisons were conducted twice in this screen to check for consistency. In some cases, mosquitoes showed no clear preference, either because mosquitoes landed in similar proportions on both socks or because the mosquitoes preferred different socks across repetitions. In those cases, additional assays were conducted as tiebreakers. This was particularly common toward the middle of the spectrum, where presumably the "attractiveness" of the various odors was similar.

Fifteen study participants provided demographic data (Fig. 4.3B). These included men and women ranging in age from 21–44. The majority of participants were Asian or



B

	937	510	GMT	846	979	419	358	939	323	950	314	288	327	265	264
gender	F	M	F	M	F	M	F	F	M	F	F	F	M	M	F
age	28	32	28	27	26	26	27	32	39	44	21	27	28	38	33
ethnicity	mixed	S.Asian	white	white	S.Asian	S.Asian	white	Asian	S.Asian	S.Asian	Asian	white	white	African	S.Asian
origin	CA	India	USA	USA	USA	India	CA	CA	India	India	CA	CA	CA	E. Africa	India
preferred?	less	more	–	more	more	less	less	more	–	more	less	more	–	–	less

Figure 4.3. The human host preference continuum in *Aedes aegypti*.
(A) Results of pairwise tests to sort participants' odors by attractiveness to mosquitoes. More preferred odors are on the left. Each curved line, ">," or "=" indicates a pairwise experiment with $n \geq 2$, with the more preferred odor placed to the left.
(B) Demographic information provided by study participants. "Preferred" indicates participants' self reports as to whether they are more, less, or equally attractive to mosquitoes than average. These categories did not predict where on the continuum participants would fall, Kruskal-Wallis $p > 0.05$.
CA: California. **USA:** United States outside California.

white. This sample size is too small to make robust conclusions, but from the data available it appears that gender, ethnicity, and region of origin had no effect on attractiveness to mosquitoes (Fig. 4.2B). Participants also performed poorly at predicting how attractive they were (Fig. 4.2B). This may be because mosquito species other than *Ae. aegypti* are common in places where these individuals have lived, with possibly different host preferences, so the participants' experiences may have no bearing on their attractiveness to *Ae. aegypti*. Alternately, individuals' self-reported attractiveness may be confounded by other factors such as itchiness of bites.

Mutations in *orco* and *Gr3* alter preference between humans

The *Ae. aegypti* genome encodes 131 members of the OR gene family, 82 of which are expressed in the adult female (Bohbot et al. 2007). ORs form heteromers consisting of an obligatory co-receptor, orco (previously designated Or7), and an additional OrX receptor that confers odorant specificity. Many mosquito ORs detect human odorants (Carey et al. 2010, Wang et al. 2010). In *Ae. aegypti*, Or4 detects the human-associated odorant 6-methyl-5-hepten-2-one and has been implicated in preference between human odor and guinea pig odor, but whether this receptor contributes to preference among humans is unknown (McBride et al. 2014).

The conserved CO_2 receptor, which also detects skin odor, consists of three subunits designated Gr1, Gr2, and Gr3; of these, Gr3 is known to be required for the receptor to function (Chapter 2, Chapter 3, McMeniman et al. 2014). Mutant mosquitoes lacking functional copies of *orco* or *Gr3* were used in follow-up tests with five

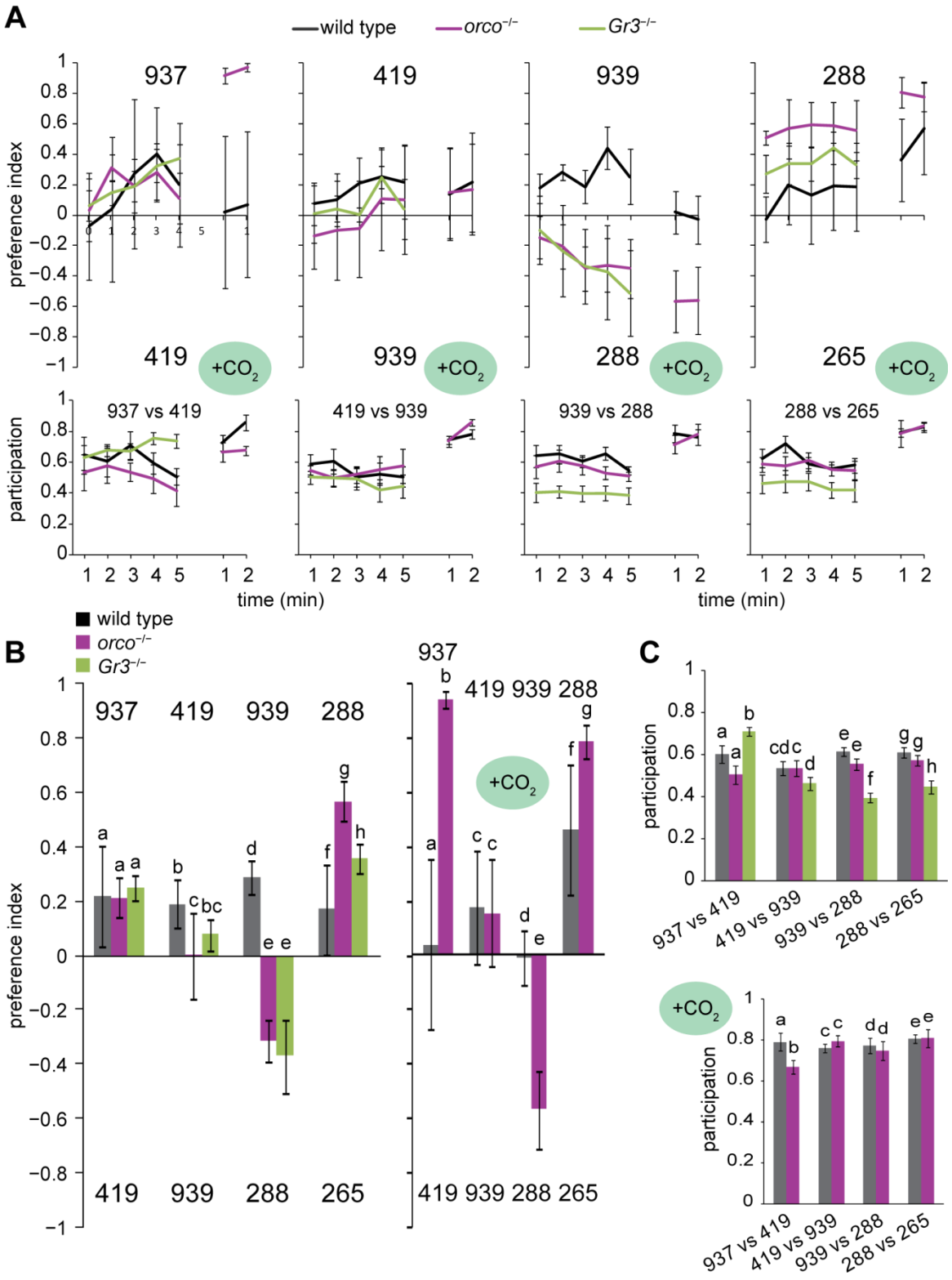

A
wild type
orco−/−
Gr3−/−
937
419
939
288
preference index
419 +CO2
939 +CO2
288 +CO2
265 +CO2
937 vs 419
419 vs 939
939 vs 288
288 vs 265
participation
time (min)
B
wild type
orco−/−
Gr3−/−
937 419 939 288
419 939 288 265
preference index
C
participation
937 vs 419
419 vs 939
939 vs 288
288 vs 265
+CO2

Figure 4.4. Preference behavior of olfactory mutant mosquitoes. (previous page)
(A) Preference index (top) and participation (bottom) measurements in tests with the indicated odors and genotypes. $n = 4–7$.
(B) Mean preference index across time points at min 2–5 without added CO_2 (left) or across time points at min 1–2 with added CO_2 (right). Each comparison was analyzed separately; nested ANOVA followed by *post-hoc* nested ANOVA with Bonferroni correction. (C) Mean participation across time points at min 2–5 without added CO_2 (left) or across time points at min 1–2 with added CO_2 (right). Each comparison was analyzed separately; nested ANOVA followed by *post-hoc* nested ANOVA with Bonferroni correction. Error bars are s.e.m. (B,C) Means with the same letter are not significantly different. Results for wild type behavior include replicates from the initial screen reported in Figure 4.3.

participants selected based on their positions on the attractiveness continuum as determined above.

The effect of genotype on observed preference varied substantially depending on where on the preference spectrum participants were located (Fig. 4.4A,B). When odor from two individuals on the more preferred end of the spectrum (person 937 and person 419) was tested in the absence of a CO_2 stimulus, both olfactory mutants preferred person 937 to the same extent as did wild type mosquitoes. However, when odor from person 419 was tested with odor from person 939, *orco* and *Gr3* mutant mosquitoes no longer discriminated between the two odors, and in tests comparing odor from person 939 with odor from person 288, the observed preference of both *orco* and *Gr3* mutant mosquitoes completely reversed from the wild type. When odors from two individuals on the less preferred end of the spectrum (person 288 and person 265) were tested, both mutants preferred person 288 to a greater extent than wild type mosquitoes. Of the two, *orco* mutants preferred person 288 to a greater extent than *Gr3* mutants.

Similar results were observed with *orco* mutant mosquitoes when assayed after a CO_2 stimulus. These mutants preferred the most attractive odor (person 937) over the second most attractive odor tested (person 419) much more strongly than wild type mosquitoes (Fig. 4.4A,B). However, they once again did not discriminate between odors from the next most preferred pair (person 419 and person 939). As was observed in the case without added CO_2, *orco* mosquitoes strongly preferred person 288 over person 939, a reversal of the wild type preference. Between the least preferred odors tested, *orco*

mutants again preferred odor 288 to odor 265 to a greater extent than wild type mosquitoes.

In pilot tests, *Gr3* mutant mosquitoes did not respond behaviorally when exposed to a puff of CO_2. This result was expected since they lack the ability to detect CO_2, and is consistent with previous results (McMeniman et al. 2014). Therefore mosquitoes of this genotype were not tested for preference behavior after a CO_2 stimulus.

The number of mosquitoes participating in each test without added CO_2 ranged from ~40–~60% across all tested genotypes (Fig. 4.4A). *Gr3* mutant mosquitoes had slightly but significantly altered participation, with fewer mosquitoes participating in trials with less preferred odors, and more mosquitoes participating in the trial with the most preferred odors of those tested (Fig. 4.4A,C). Participation rates increased in both wild type and *orco* mutant mosquitoes when they were exposed to a puff of CO_2. Participation rates did not differ between wild type and *orco* mutant mosquitoes, either with or without a CO_2 stimulus (Fig. 4.4A,C).

Neuronal responses to foot odor associated with *orco* and *Gr3*

The summed responses of antennal neurons to foot odor collected on glass beads as in Chapter 2 and measured by electroantennograms (EAG), were not significantly different in wild type or *orco* mutant mosquitoes (Figure 4.5A). Thus, any changes in neuronal response attributable to OR activity fall below the threshold of detection.

The cpA neuron on the maxillary palp expresses Gr1, Gr2, and Gr3 and detects both CO_2 and skin odor (Kellogg 1970, Tauxe et al. 2013). CpA responses to human odor have been previously observed to vary among individuals (Chapter 2). To

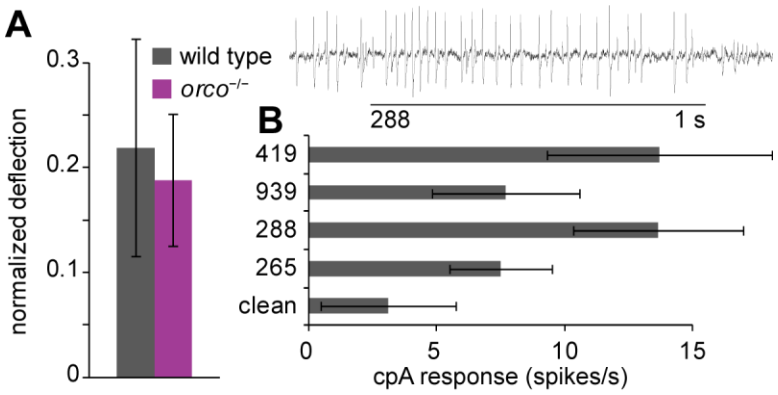


Figure 4.5. Neuronal responses to human odor.
(A) EAG responses to human odor from glass beads (mixed odor from GMT and person 265).
$n = 7$, t-test $p > 0.05$.
(B) Representative trace and average cpA responses to odor from socks worn by the indicated participants.
$n = 6–10$. ANOVA, $p > 0.05$. Error bars are s.e.m.

determine if cpA responses to odors used in behavioral tests vary, air was puffed through worn socks and cpA responses were recorded. No significant differences were observed in cpA responses to odors from different individuals, or even between worn socks and a clean control sock. (Fig. 4.5). There is a trend for odors from worn socks to evoke more cpA activity than clean socks, and this trend may become significant with additional replicates. It is worth noting that previous observations used odor collected on glass beads rather than socks (Chapter 2), and this may be a more effective method for presenting odor for electrophysiology.

Discussion

The olfactory GRs of the cpA neuron are known to mediate activation of host-seeking behavior (reviewed in Chapter 1), but have not previously been implicated in host preference. At the spatial scale of the cage assay used in this study, *Gr3* mutants show no deficits in finding a host (McMeniman et al. 2014), which is consistent with the observed high participation of *Gr3* mutants in this study. These mutants did have reduced participation in tests between individuals known to be less attractive to wild type

mosquitoes, consistent with a Gr3-mediated deficit in activation that can be overcome by the presence of other attractive cues at short range.

Unlike GRs, ORs have previously been shown to mediate preference in *Ae. aegypti* for human odor over guinea pig odor, and this has been attributed to the activity of Or4 (DeGennaro et al. 2013, McBride et al. 2014). Or4 sensitively detects 6-methyl-5-hepten-2-one, which is much more abundant in human odor than in the odor of several other host animals (McBride et al. 2014). However, 6-methyl-5-hepten-2-one has been found to be more abundant in the odor of less preferred humans (Logan et al. 2008), so it may be that Or4 has a different effect on preference among otherwise acceptable human odors.Both *orco* and *Gr3* mutants had significantly different preference behavior from wild type mosquitoes, and the nature of the difference changed depending on how attractive the odors being compared were.

Both mutants discriminated between two attractive odors equally well as wild type mosquitoes in the absence of added CO_2, but had increasingly altered preference behavior when tested with less and less preferred odors such that their preference between two less attractive odors was the opposite of wild type. With the addition of a CO_2 stimulus, *orco* mutants preferred a highly attractive sock even more strongly than wild type mosquitoes, but again this preference was reversed when mosquitoes were tested with two less attractive odors. Preference of both mutants reversed again with the least attractive odors tested, with both mutants preferring the more attractive sock significantly more than wild type mosquitoes.

The odor-dependent nature of the behavioral phenotype of both *Gr3* and *orco* mutations argues for the involvement of multiple odors and receptor pathways in preference behavior. One model that could explain the observed differences in behavior is that mutant mosquitoes have deficits in detecting some attractive odorants, resulting in reduced preference for attractive odors, but that this can be overcome when attractive odorants they do detect are present or abundant in the most attractive odors. It is also possible that mutant mosquitoes have a deficit in their ability to detect repellent odorants present in less attractive odor, or that the salience of some odor components is increased when other odorants are no longer detected. It is not yet possible to discriminate among these possibilities, but the clear behavioral phenotypes indicate that this is due to limitations in the electrophysiological methods used so far.

The observed preference behaviors of *orco* and *Gr3* mutants were similar to each other, even when they were clearly different from wild type behavior. This was unexpected, since the receptors they lack are expressed in different sets of neurons. It is possible that the same attractive or repellent odorants are detected by both pathways: many of the same odorants are detected by the GR-expressing cpA neuron and by various ORs in *Anopheles gambiae* (Carey et al. 2010, Wang et al. 2010, Tauxe et al. 2013), and this is also likely in *Ae. aegypti*, although this by itself is insufficient to explain why both mutations have such similar behavioral phenotypes. CpA activity is known to increase the salience of other, non-CO_2 odorants (Dekker et al. 2005), so it may be that wild type behavior depends on activation of both pathways.

There is a third family of chemoreceptors in insects, the ionotropic receptors (IRs), which are typically expressed in separate neurons from ORs or GRs and do not require orco or Gr3 function. Given the high participation of all genotypes across all odors tested in this assay, it is clear that *orco* and *Gr3* mutant mosquitoes still detect and are attracted to human odor in this context. This residual olfactory behavior may be because OR- and GR–mediated olfactory pathways are partially redundant, and/or it may be due to the contributions of IRs. While no mosquito IRs have yet been decoded, olfactory *Drosophila* IRs frequently detect polar compounds such as amines and carboxylic acids (Croset et al. 2010, Liu et al. 2010), and the same is probably also true for mosquito IRs. Thus L-lactic acid, which has been implicated in host preference in *Ae. aegypti* (Steib et al. 2001, reviewed in Chapter 1), is likely detected by IRs. The relative contribution of IRs in the context of this assay remains to be determined.

It is hoped that the data presented here will inform future studies to determine which human odorants contribute to host preference at each part of the attractiveness spectrum, and how different olfactory pathways interact to determine preference behavior.

Methods

Mosquitoes

Wild type mosquitoes were from the Orlando strain,s a selected laboratory strain that was probably established in Orlando, FL, in 1939 or 1942 (Kuno 2010). Mutant mosquitoes were created in an Orlando background and provided by the L. B. Vosshall

laboratory (Rockefeller University; DeGennaro et al. 2013, McMeniman et al. 2014). The *orco* mutants in the experiment described in Fig. 4.2A were homozygous for the $orco^{16}$ loss of function allele; *orco* mutants in other experiments were heterozygous for the $orco^{5}$ and $orco^{16}$ alleles. Both alleles have frameshift mutations of differing lengths in the same location; no differences were observed in behavior between alleles. *Gr3* mutants were homozygous for the $Gr3^{ECFP}$ allele described by McMeniman et al. (2014).

Ae. aegypti of all genotypes were reared at 27°, 70% relative humidity, and L:D 14:10. Larvae were fed on alfalfa pellets; adults were fed 10% sucrose solution. Colonies were maintained by females bloodfed on bovine blood provided through a membrane feeder. Females used in experiments were housed with males and were not bloodfed.

Odor-laden socks

Human odor was collected on nylon shoe liner–style socks (Target) worn inside cotton or polyester socks for ~6 hr. Socks were used in experiments the same day they were worn. Volunteers washed their feet with fragrance-free soap (pure glycerine soap, The Soap Works) and water immediately before putting on the socks for odor collection, and both sets of socks were laundered between uses with fragrance-free detergent (Tide). The same socks were returned to each volunteer for re-use. Participants were asked to wear the same shoes and maintain a similar routine (including diet and exercise habits) for each collection day.

Two-choice cage assay

Trials were conducted between 14:00 and 19:00 at 27°C and 30–45% relative humidity. Twenty to thirty 6–14-day-old female *Ae. aegypti* were starved overnight and allowed to acclimatize undisturbed in the assay cage for at least 10 min before the beginning of each assay. The cage measured 30 cm × 30 cm × 30 cm with mesh sides and a glass top, and was separated from the experimenter by a transparent partition. Light from overhead fluorescent bulbs was balanced with reflective white paper around the cage and the arrangement of socks was alternated to avoid side bias. Each cage of mosquitoes was assayed no more than once/hr. Socks (odor-laden or clean) were hung from either side of the cage from binder clips attached to the glass top, and mosquito behavior was video recorded for 5 min. To determine how behavior changed after addition of a CO_2 stimulus, at the end of the 5 min assay period, the experimenter exhaled through a tube whose mouth was located near the cage on a side without any sock; behavior was subsequently recorded for another 2 min. Cages, equipment, and socks were handled with nitrile lab gloves and washed with fragrance-free detergent (Tide) between uses to reduce odor contamination. Preference index was calculated at 1 min time intervals during the assay as (# mosquitoes on side of cage with sock A − # mosquitoes on side of cage with sock B) / (total # mosquitoes on both sides with socks), excluding resting mosquitoes.

One trial with unusually low participation (<30%) was excluded from the experiment in Fig. 4.2A. The same cage of mosquitoes had high participation in other trials conducted the same day, so the change in participation was attributed to a loss of

odor from the socks being tested. To mitigate this issue, socks used in other experiments were stored in plastic bags (Zip-Loc) between trials and socks from both feet of each participant were used in sequence.

Electrophysiology

Single-sensillum recordings. 7-day-old adult female mosquitoes were used for recordings. Each mosquito was restrained on a microscope slide with its head propped up and stuck to an elevated cover slip with double-stick tape (3M). The maxillary palp was gently brushed with tape to remove scales and pressed into tape on the cover slip with a blunt glass needle to keep it from moving. A reference electrode, consisting of a silver wire electrode inserted into a glass micropipet filled with sensillum lymph ringer (Kaissling and Thorson 1980), was inserted into the insect's eye. A recording electrode, consisting of a silver wire electrode inserted into a glass micropipet filled with sensillum lymph ringer, was inserted into the base of a capitate peg sensillum using a micro-manipulator under magnification.

To create odor cartridges from worn nylon socks, the elastic around the outside of the sock was removed and the nylon cut lengthwise in half; each half was inserted into a 25 ml disposable serological pipet capped with a blue tip. A constant 8 ml/s stream of carbon filtered room air was switched from a cartridge with a clean sock to the odor cartridge using a Syntech CS-55. The resulting airflow was delivered into a glass tube with a constant, humidified airstream (10 ml/s) whose mouth was centered on a ~1 cm from the mosquito head. Each cartridge was used $\leq$ 2 times.

Signals were amplified 1000× and band-pass filtered to admit signals between 10 Hz–1.0 kHz with a Iso-Dam amplifier (World Precision Instruments). Signals were digitized with a Digidata 1440 (Molecular Devices). Recordings were analyzed in the AxoScope and pClamp programs (Molecular Devices); spikes (i.e., action potentials) were counted manually. Neuronal responses were corrected for baseline firing rate: reported firing frequencies were calculated as (number of spikes during 1 s stimulus presentation) – (number of spikes during 1 s prior to stimulus presentation).

Electroantennography (EAG). Decapitated heads of adult female mosquitoes (4–14 days old) were used for recordings. A reference electrode consisted of a silver chloride–coated silver wire inserted into a glass capillary filled with Beadle-Ephrussi ringer (Benton and Dahanukar 2010). The capillary was sealed at the distal end with a pore blown in the side, into which the neck tissue of the mosquito head was inserted. The tip of the antenna was removed using a scalpel to cut through the distalmost flagellomere and a recording electrode, consisting of a silver chloride–coated silver wire inserted into a saline-filled micropipet, was placed over the cut end.

Human odor was collected on glass beads worn in socks for use in EAG recordings. Volunteers inserted 10–13 ml craft beads (size 10/0; Michaels) into each of their socks so that the beads were brought into contact with the toes and sole of the foot for ~6 hr of normal activity. To reduce extraneous odors, volunteers washed their feet with fragrance-free soap (Dove) and water immediately before odor collection, and socks were laundered between uses with fragrance-free detergent (Tide). Odor-laden beads were provided by GMT and person 265; these were mixed together, and 20 ml beads

were placed inside a 25 ml disposable serological pipet capped with a blue tip and sealed with Parafilm between uses. A constant 8 ml/s stream of carbon filtered room air was switched from a cartridge with a clean sock to the odor cartridge using a Syntech CS-55, as described above for stimulation with odor-laden socks.

EAG signals were amplified 100× and band-pass filtered to admit signals from 0.1 Hz–10.0 kHz. Maximum deflections evoked by odor stimuli were normalized to interspersed pulses of a reference odorant (acetic acid diluted at 10^{-1} in water) according to the formula: Raw response (mV)/($ax + by$), where a and b are the responses (mV) to the previous and subsequent reference odor stimulation, respectively, and x and y are the proportion of time elapsed between stimuli (so that $x + y = 1$). Responses to the reference odorant did not differ between genotypes.

Statistics

All statistical analyses were performed in R. Electrophysiological data were recorded with one replicate per insect.

Ethics

Use of animals for feeding mosquitoes was monitored by the Institutional Animal Care and Use Committee at UCR and conducted in accordance with protocol A-2010023 issued to Ring Cardé and Anandasankar Ray.

Use of human subjects was monitored by the Human Research Review Board at UCR and conducted in accordance with protocol HS 14-017 issued to Anandasankar Ray.

Chapter 5. Conclusions and future challenges.

Mosquitoes and the diseases they carry are a global problem. There are hundreds of millions of cases of malaria every year, all transmitted by *Anopheles* mosquitoes, and even though a concerted global effort is helping to reduce this number, half the world's population remains at threat and hundreds of thousands of people still die every year of this disease (Murray et al. 2012, WHO 2014a). Dengue, transmitted primarily by *Aedes aegypti* (L.) and also by *Aedes albopictus* (Skuse), threatens a similar proportion of the world's populations, mostly in tropical and subtropical urban centers (Bhatt et al. 2013). In 2013, there were over two million cases of dengue in the Americas alone, including about 40,000 cases of the severe form, dengue hemorrhagic fever (DHF), and the numbers are increasing worldwide (WHO 2014b). Dengue is prone to explosive epidemics, and DHF is the leading cause of severe illness and death in children in several regions where it is endemic (WHO 2014b). Chikungunya virus, also transmitted by *Ae. aegypti* and *Ae. albopictus*, is an emerging disease that went from the first recorded case in the Americas on the Caribbean island of St. Martin in early December 2013 to >750,000 suspected cases by October 2014 (WHO 2014c).

The increasing efficiency and quantity of global trade and travel has facilitated the movement of mosquitoes and mosquito-borne diseases across the globe, particularly invasive *Aedes* species. Warmer global temperatures may also be playing a role in increasing the geographic range of habitat for certain mosquito species. The yellowfever mosquito *Ae. aegypti* spread from Africa around the globe by hitchhiking along human trade routes centuries ago and has become naturalized in many parts of the tropics,

including the Americas (Brown et al. 2011). The Asian tiger mosquito *Ae. albopictus* is a more recent invader and has been spreading globally by means of commercial goods including automobile tires and ornamental plants. In the United States, this mosquito was first detected in Texas in 1985, when it had already been spread by human transport of used tires throughout the eastern United States (Moore and Mitchell 1997), and has since continued to spread. Once established, these mosquitoes can initiate local transmission of diseases such as dengue and chikungunya viruses after coming into contact with viremic travelers.

West Nile Virus, which is transmitted primarily by *Culex* mosquitoes, was introduced into New York City in 1999, swept through bird and mosquito populations, and is now endemic (and enzootic) throughout most of North and Central America. The ranges of four serotypes of dengue virus have spread to cover most of the tropics in the last 30–40 years, and many areas are now threatened by more than one serotype (Messina et al. 2014). This is of particular concern since if someone survives a bout of dengue but is later infected by a second serotype, the risk of DHF and death increases (WHO 2014b). Dengue virus was reintroduced into the Florida Keys in 2009 after having been considered eliminated for more 70 years (CDC 2010); it has now spread into southern Florida, including the Miami area. Chikungunya virus, which has similar symptoms to dengue and is vectored by the same mosquitoes, has gone from a relatively minor tropical disease to a worldwide threat. Locally transmitted cases were reported in Florida this July (Kendrick et al. 2014), and an infected *Ae. aegypti* mosquito was discovered in Texas in August, indicating that the disease could become resident in the United States.

In California, *Ae. albopictus* became established in the city of El Monte in Los Angeles County in 2001 and has since spread to 13 neighboring cities. In 2013, populations of *Ae. aegypti* were discovered in several cities in Madera, Fresno, and San Mateo Counties (Gloria-Soria et al. 2014), and in 2014 additional populations were discovered in Kern, Tulane, and San Diego Counties. A third species, *Aedes notoscriptus*, known as the striped or backyard mosquito in its native Australia, was discovered in the cities of Monterey Park and Montebello, Los Angeles County (personal communication, M. E. Metzger, California Department of Public Health).

There is a critical need for new strategies to combat mosquito-borne disease, particularly in developing countries that bear a disproportionate amount of the global disease burden. The *Plasmodium* parasites that cause malaria have a complex life cycle and present different antigens at each stage, complicating vaccine development (Enayati and Hemingway 2010). Therapeutics are available, including chloroquine and its derivatives and artemisinin, but resistance is a major and increasing problem. The *Anopheles* mosquitoes that transmit *Plasmodium* are likewise resistant to many insecticides, complicating vector control (Enayati and Hemingway 2010). Dengue also presents problems for vaccine developers: since exposure to one serotype increases the likelihood of severe disease when a person is infected with a second serotype, a "tetravalent" vaccine must immunize patients against at least all four of the widespread serotypes simultaneously to be practical (Webster et al. 2009, Sabchareon et al. 2012). The efforts to produce such a vaccine have been complicated by the discovery of a fifth

serotype (Normile 2013). There are no therapeutic treatments for dengue, so prevention is the only effective control measure available.

Yet for all of their global impact and deadly effect, mosquito-borne diseases have a critical weakness: the mosquito. In the absence of other effective measures to stop disease transmission, public health agencies use mosquito control measures that range from the traditional (e.g., adulticides, larvicides, insecticide-treated bednets, indoor residual spraying) to more newfangled techniques. Emerging technologies include releasing sterile males that compete with wild males for access to females and thus reduce overall reproductive success in the population. Variations on this sterile insect technique (SIT) in current use include the release of (transgenic) insects carrying a dominant lethal gene (RIDL), developed by Luke Alphey and others at Oxitec (Alphey et al. 2010) or releasing male mosquitoes that carry a sterility-inducing symbiont such as *Wolbachia*, which induces cytoplasmic incompatibility and thus kills the male's offspring if the female he mates with does not also carry *Wolbachia* (Bourtzis et al. 2014). Another strategy is to replace wild populations with mosquitoes that carry natural symbionts such as *Wolbachia* or transgenically modified gut bacteria that make them unable to transmit disease (Coutinho-Abreu et al. 2010, Hoffmann et al. 2011, Walker et al. 2011, Bian et al. 2013).

Chemicals that interrupt host-seeking behavior have potential to mask human hosts, repel mosquitoes away from humans, or attract mosquitoes to traps for surveillance or population control. All of these tactics can be used by themselves or to complement other strategies like those described above. Existing repellents have major drawbacks:

DEET is expensive, has an unpleasant texture and odor, and is not compatible with synthetic fabrics; analogs like IR3535 and picaridin are likewise expensive and not frequently available; and botanicals including citronella and eucalyptus oils are only effective at high concentrations for short periods of time. Because a mosquito must succeed in obtaining a blood meal at least twice to transmit disease, any reduction in human biting success due to more effective or more frequently used repellents and/or masking agents will have a multiplied effect in disease control.

Current traps used for mosquito and vector-borne disease surveillance also have major limitations. Passive traps such as resting boxes are used routinely for disease surveillance; these attract some mosquitoes, but not typically anthropophilic species (those that specialize on feeding on humans), which are the most important vectors of human disease, including malaria, dengue, and chikungunya. Standard traps for human malaria mosquitoes require both a carbon dioxide (CO_2) source to attract mosquitoes to the vicinity of the trap and a fan to suck them into the trap; both of these are difficult to obtain in the field in underdeveloped regions where surveillance is most needed. For disease-transmitting *Aedes* species, including *Ae. aegypti* and *Ae. albopictus*, specialized traps such as ovitraps, BioGents Sentinel traps, and CDC autocidal gravid ovitraps greatly increase detection success over traditional light traps, CO_2, and gravid traps (Barrera et al. 2014; personal communication, M. E. Metzger, California Department of Public Health). However, even specialized traps have mixed success in early detection of invading mosquitoes in urban environments, especially when populations are low. Public health officials in California currently employ a variety of different traps to monitor

populations of invasive *Aedes* mosquitoes, but reports of day-biting mosquitoes often precede trap captures in new areas (personal communication, M. E. Metzger, California Department of Public Health). In Los Angeles County, the use of only standard CO_2, and gravid traps for arbovirus surveillance failed to detect an established population of *Ae. albopictus* for nearly a decade (Zhong et al. 2013).

Aedes aegypti is an emerging genetic model for complex behavior

To improve on existing host-seeking disruption technologies, it is helpful to have a better understanding of how host-seeking actually works. Several features of *Ae. aegypti* make it a particularly good species to work with for studying this behavior. First, since its "wild" habitat is typically in and around human homes, it is preadapted for living in the laboratory. Its stenogamous mating habits and straightforward feeding requirements make it relatively easy to rear, and its egg diapause allows eggs to be stored for months at a time. This last feature greatly simplifies maintaining multiple strains or genotypes with reduced risk of cross-contamination.

Second, *Ae. aegypti*'s host-seeking behavior is easily replicated in the laboratory. This species is active during the day and an aggressive biter of humans, so relatively easy for most researchers (i.e., day-active humans) to work with. Despite this convenience, the sophistication of host-seeking behavior, with multiple pathways leading to the same end result of mosquitoes reaching hosts, means that until very recently it has been difficult to identify specific odorants that are involved in each stage of the behavior.

Finally, transgenic tools are increasingly available for use in this species that can be used to dissect behaviors. When I started working in this system in 2009, very few

technologies were available to transform mosquitoes or create targeted mutations, and these required an impractical amount of time and money. Instead, I developed a procedure to take advantage of the discovery that butyryl chloride selectively inhibits the mosquito CO_2 receptor for hours at a time. This chemical method allowed me to elucidate how human odor residues trigger mosquito host-seeking behavior even in the absence of CO_2 (Chapter 2).

In the meantime, advances in transgenic technology have made it possible for mutants to be generated for key olfactory genes. This effort has been led by the L. B. Vosshall laboratory at Rockefeller University, and studies with the mutant lines they have created have led to interesting insights in how different olfactory pathways contribute to behavior (DeGennaro et al. 2013, McMeniman et al. 2014, Chapter 4). Among other observations, it is becoming inescapably obvious what careful observers of mosquito behavior have suspected for decades: host-seeking behavior is not a singular process, but involves different sets of likely redundant pathways and odorants at each stage of behavior (Chapter 1). With the advent of the latest generation of targeted genome editing technologies like TALENs and CRISPR, it is now easier than ever to generate mutants in mosquitoes and other organisms, and unlike some other mosquito species, *Ae. aegypti* can be readily transformed using these tools.

The CO_2-sensitive cpA neuron triggers subsequent host-seeking behaviors

CO_2 is a key cue used by many mosquito species in locating hosts, but its actual role in host-seeking behavior has been debated. The essence of the debate comes down to a dichotomy between laboratory studies which show that mosquitoes of several species

will rapidly fly upwind and locate a source of CO_2, and field studies which show that a CO_2-baited trap is much less effective at attracting anthropophilic species than a human, or even a trap baited with CO_2 along with synergistic odors like lactic acid or 1-octen-3-ol (reviewed in Chapter 1). It has long been known that CO_2 activates mosquitoes, that is, triggers resting mosquitoes to take flight (Daykin et al. 1965), and in moving air and in the absence of other host cues, CO_2 triggers mosquitoes to fly upwind (Kennedy 1939). There is now a strong body of evidence, including work from the Vosshall and Cardé laboratories as well as the work presented in this dissertation, that both of these behaviors are mediated by the GR-expressing cpA neuron class located on the maxillary palps, and that subsequent host-seeking behaviors are not.

As one might expect, mutant mosquitoes that lack functional cpA neurons no longer activate when exposed to a CO_2 stimulus, and this prevents them from responding to other, isolated host cues such as heat or odor (McMeniman et al. 2014). Likewise, after activation by CO_2, their responses to skin odor are greatly enhanced (Dekker et al. 2005). The same cpA neuron also detects components of the skin odor blend, and rendering it nonfunctional also prevents mosquitoes from activating in response to host odor without added CO_2 (Chapter 2). The detection of both CO_2 and host odors is mediated by the GRs expressed in the cpA neuron, Gr1, Gr2, and Gr3. Knocking out Gr3 prevents detection of both CO_2 and other odorants (McMeniman et al. 2014, Chapter 3), and Gr2 and Gr3 together make a functional receptor whose function is modulated by Gr1 (Chapter 3).

After activation, mosquitoes are more responsive to other cues than to CO_2, to the point that they will leave a CO_2 plume and follow a plume of foot odor instead (Lacey et al. 2014). Even when cpA is nonfunctional, mosquitoes that randomly take flight are quite capable of finding a host odor source, presumably relying on other olfactory pathways (Chapter 2, McMeniman et al. 2014). In small-scale experiments with mosquitoes in cages 30 cm on a side, these mosquitoes show hardly any deficits at all in navigating to complex host cues such as a worn sock or a live mouse (Chapter 4, McMeniman et al. 2014). These disabled mosquitoes likewise show normal landing and probing behaviors on human odor sources (Chapter 2, Chapter 4). Current data suggest that *Gr3*-disabled mosquitoes, as well as *orco* mutant mosquitoes, may have altered host preferences (Chapter 4). If this is confirmed, it will be a novel function of the GR/cpA pathway and may lead to interesting insight into the mechanisms that drive preference.

Frontiers in mosquito olfaction and host-seeking behavior

Mosquitoes lacking functional odorant receptors (ORs) due to a loss of function in the obligate OR co-receptor orco show deficits in navigating toward honey but surprisingly few deficits in their host-seeking behaviors (DeGennaro et al. 2013). The ionotropic receptors (IRs) are another class of receptors that is likely involved in mosquito responses to host odors. In other species, these receptors detect polar compounds such as acids, aldehydes, and amines, many of which attract mosquitoes (reviewed in Chapter 1). Targeted mutations in genes coding for these receptors will help elucidate how they contribute to host seeking.

Most IRs, indeed most mosquito receptors in general, are expressed on the antennae. Antennal sensilla are relatively inaccessible to single sensillum recordings (personal observation), and most of them have not been functionally characterized. The antennal lobe (AL) is the central clearinghouse in the brain for olfactory input and is innervated by olfactory neurons from the antenna as well as the maxillary palps and proboscis (Ignell et al. 2005). Functional imaging of the AL, facilitated by calcium-responsive fluorescent markers like GCaMP, will be a key technique for decoding odor responses of the full receptor repertoire, as well as the more limited repertoires of mosquitoes with olfactory receptor mutations.

The human odor blend is highly complex, and although many components have been identified, their relative abundances are not well known. Once olfactory pathways are identified that determine preference, gas chromatography (GC) of host odor linked with antennal imaging can be used to determine which individual components are responsible for driving behaviors.

Once the odors involved at different stages of host-seeking and the mosquito olfactory pathways that detect them are identified, it will be possible to investigate the microbial or biochemical origins of those odors and how they vary among people. Those odors and pathways will be prime targets for new mosquito control technologies.

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Appendix 1. Non–human-associated ligands of cpA.

A 65-odor panel was tested for cp activity in *Ae. aegypti* in 2010 using the same methods as described in Chapter 2. Odors were selected for commercial availability and structural similarity or identity with known ligands of *An. gambiae* cpA (Lu et al. 2007), *Cx. quinquefasciatus* cpA, and *D. melanogaster* ab1C (Turner and Ray 2009), *Cx. quinquefasciatus* cpB (Syed and Leal 2007), and *An. gambiae* cpB and cpC (Lu et al. 2007). Many of these odorants interacted with cpA; those associated with human odor are reported in Fig. 2.2, others in Fig. A1.1A. Dose-dependent responses were observed for several representative odorants (Fig. A1.1B). Additionally, butyryl chloride was identified as a long-term inhibitor of cpA (detailed in Chapter 2).

No odors were identified that activated or inhibited cpB. In *Ae. aegypti*, this neuron expresses Or49, which is not homologous with either of the Ors expressed in *An. gambiae* capitate peg neurons. Results for cpC were inconclusive, as the first odor in the panel that interacted with this neuron was 1-octen-3-ol, which at the 1% concentration used superactivated the neuron and prevented it from responding to other odorants. Similar phenomena have been observed in other neurons (Montague et al. 2011, Turner et al. 2011). This neuron expresses Or8, which is homologous with the receptor expressed in the cpB neuron of other mosquitoes (Lu et al. 2007).

A small number of additional odorants were tested for cpA in *Ae. aegypti* or *An. gambiae* in other contexts. These are included in Fig. A1.1A and A1.1C.

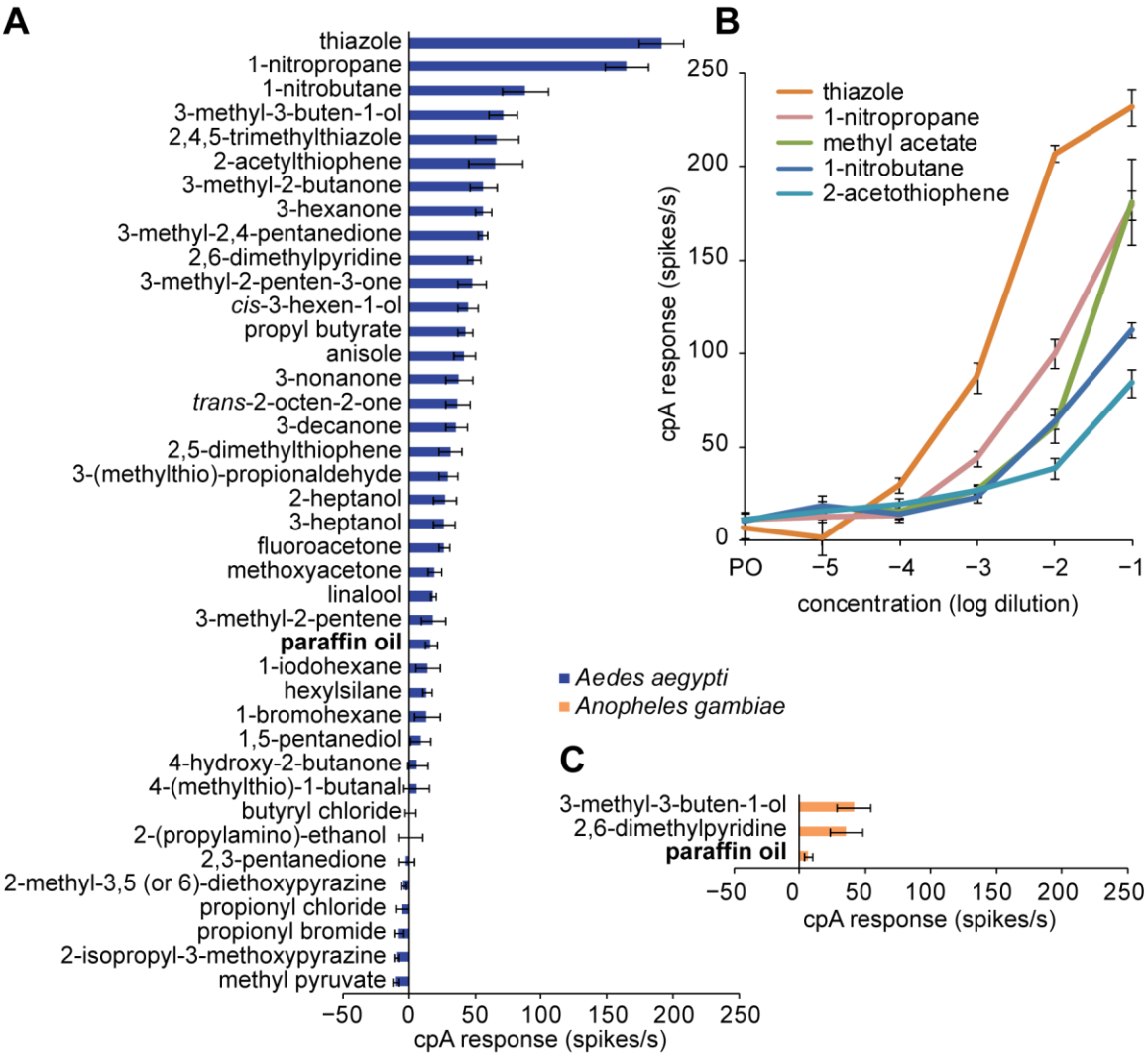

Figure A1.1. cpA responses to additional odorants.
(A) Mean responses of the cpA neuron in *Ae. aegypti* to 0.5 s pulses of odorants not known to be present in human odor. n = 2–13, see Appendix 3 for details.
(B) CpA dose responses in *Ae. aegypti* to representative odorants diluted in paraffin oil. n = 5.
(C) Mean responses of the cpA neuron in *An. gambiae* to 0.5 s pulses of odorants not known to be present in human odor. n = 9, see Appendix 3 for details.
(A,C) All odorants were diluted in paraffin oil at 10^{-2}. Error bars are s.e.m.

Appendix 2. Human-associated odorants.

Table A2.1. List of tested human-associated odorants and bibliography.

Odor name	CAS	Where previously detected	References
1-butanol	71-36-3	Back/forearm skin, forehead sweat (fresh and incubated), foot microbe headspace, total effluent	1–5
1-chlorohexane	544-10-5	Handprints, breath	6–8
1-decanol	112-30-1	Incubated forehead sweat	2
1-hepten-3-ol	4938-52-7	Handprints	6,7
1-hexanethiol	111-31-9	Total effluent	5
1-hexanol	111-27-3	Incubated forehead sweat, total effluent	2,5
1-nonanol	143-08-8	Incubated forehead sweat	2
1-octanol	111-87-5	Incubated forehead sweat, breath	2,8
1-octen-3-ol	3391-86-4	Back/forearm skin, handprints, forehead/trunk sweat	1,6,9
1-pentanol	71-41-0	Forehead sweat (fresh and incubated), total effluent	2,5
1-pentene	109-67-1	Total effluent	10
2,3-dimethyl-2-cyclopenten-1-one	1121-05-7	Foot microbe headspace	4
2,5-dimethylpyrazine	123-32-0	Foot microbe headspace	4
2-ethyl-3,6-dimethylpyrazine	55031-15-7	Foot microbe headspace	4
2-furoic acid	88-14-2	Incubated axillary sweat (as methyl ester)	11
2-hexanol	626-93-7	Back/forearm skin	1
2-hexene	7688-21-3	Total effluent	5
2-hexenoic acid	13419-69-7	Female apocrine sweat	12
2-methyl-1-butanol	137-32-6	Incubated forehead sweat, foot microbe headspace	2,3
2-methyl-3-heptanone	13019-20-0	Breath	8
2-methylbutyraldehyde	96-17-3	Handprints, foot microbe headspace	3,6,13
2-methylbutyric acid	116-53-0	Foot and skin microbe headspace	3
2-methylcyclopentanone	1120-72-5	Back/forearm skin	1
2-methylheptanoic acid	1188-02-9	Male and female axillary sweat	12,14
2-methylhexanoic acid	4536-23-6	Male and female axillary sweat, female apocrine sweat	12,14
2-methylnonanoic acid	24323-21-5	Male and female axillary sweat, female apocrine sweat	12,14
2-nonanone	821-55-6	Incubated forehead sweat, foot microbe headspace	2,4

Odor name	CAS	Where previously detected	References
3,5,5-trimethyl-1-hexanol	3452-97-9	Incubated forehead sweat	2
3-acetoxy-2-butanone	4906-24-5	Foot microbe headspace	4
3-hexanol	623-37-0	Back/forearm skin	1
3-methyl-1-butanol	123-51-3	Incubated forehead sweat, foot and skin microbe headspace	2–4
3-methyl-2-buten-1-ol	556-82-1	Incubated forehead sweat	2
3-methylcyclopentanone	1757-42-2	Back/forearm skin	1
4-heptanone	123-19-3	Total effluent	5
4-methyl-3-penten-2-one	141-79-7	Fresh forehead sweat	2
5-nonanone	502-56-7	Foot microbe headspace	4
6-methyl-5-hepten-2-ol	1569-60-4	Incubated forehead sweat	2
acetoin	513-86-0	Fresh and incubated forehead sweat, foot microbe headspace	2–4
acetone	67-64-1	Back/forearm skin, fresh forehead sweat, axillary headspace, breath, total effluent	1,2,5,10, 15,16
acetophenone	98-86-2	Back/forearm skin, foot microbe headspace	1,4
adipic acid	124-04-9	Handprints	6
benzaldehyde	100-52-7	Incubated axillary sweat, microbial headspace, back/forearm skin, handprints, foot skin and headspace, total effluent	1,4,6,11, 17,18
benzothiazole	95-16-9	Back/forearm skin, foot headspace, breath, total effluent	1,5,16,18
butanedione	431-03-8	Foot microbe headspace	3
butanone	78-93-3	Handprints, total effluent	5–7,10
butyric acid	107-92-6	Back/forearm skin, fresh and incubated forehead sweat, forehead/trunk sweat, leg sweat, whole body sweat	1,2,9,19,20
cyclohexanone	108-94-1	Fresh forehead sweat, foot microbe headspace, total effluent	2,4,5
dimethyl disulfide	624-92-0	Handprints, foot microbe headspace	4,6
ethyl formate	109-94-4	Total effluent	10
ethyl valerate	539-82-2	Breath	8
heptanoic acid	111-14-8	Male and female axillary sweat, female apocrine sweat, handprints, leg sweat	6,12,14,19
hexanal	66-25-1	Fresh forehead sweat, axillary headspace and incubated sweat, foot skin and headspace, handprints, arm and leg skin	2,11,13,15, 18,21
hexanoic acid	142-62-1	Forehead/trunk sweat, back/forearm skin, male and female axillary sweat, female apocrine sweat, handprints, leg sweat,	1,2,6,9,12, 14,19,20

Odor name	CAS	Where previously detected	References
hexanoic acid (cont'd)		whole body sweat	
indole	120-72-9	Incubated forehead sweat, foot microbe headspace, handprints, total effluent	2,4,6,7,17
isobutyric acid	79-31-2	Forehead/trunk sweat, whole body sweat	9,20
isovaleric acid	503-74-2	Forehead/trunk sweat, back/forearm skin, incubated forehead sweat, foot and skin microbe headspace, whole body sweat	1–4,9,20
methanol	67-56-1	Breath, total effluent	5,8,10
methyl acetate	79-20-9	Total effluent	5,10
naphthalene	91-20-3	Incubated axillary sweat, foot microbe headspace, breath, total effluent	4,8,11,17
octanal	124-13-0	Axillary sweat, arm, leg, and back skin, fresh forehead sweat, foot skin and headspace, handprints, worn clothing, total effluent	1,2,6,11, 13,15,17, 18,21–23
octanoic acid	124-07-2	Forehead/trunk sweat, back/forearm skin, male and female axillary sweat, female apocrine sweat, leg sweat, foot headspace handprints, total effluent	1,6,9,12, 14,17–19
phenylacetaldehyde	122-78-1	Handprints	13
propionaldehyde	123-38-6	Handprints, total effluent	5,6
propionic acid	79-09-4	Forehead/trunk sweat, back/forearm skin, whole body sweat, handprints, total effluent	1,2,6,9,10, 20
pyrazine	290-37-9	Foot microbe headspace, handprints	4,6
pyridine	110-86-1	Incubated axillary sweat, back/forearm skin, fresh and incubated forehead sweat, handprints	1,2,6,7,11
pyruvic acid	127-17-3	Leg sweat, total effluent	5,24
trimethylpyrazine	14667-55-1	Foot microbe headspace, handprints	4,6

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Appendix 3. Summary of cpA responses to non-CO_2 ligands.

Table A3.1. 65-odor panel (Fig. 2.2, Appendix 1) in *Ae. aegypti* (Rockefeller strain) cpA

All odorants diluted at 10^{-2} in paraffin oil.

odorant	abbrev.	mean response (spikes/s)	s.e.m.	*n*	human odor
pyridine	prd	191.75	15.85	4	•
thiazole	thz	191.00	16.84	13	
1-nitropropane	3NO	164.50	16.28	4	
cyclohexanone	c6on	136.00	8.80	10	•
1-nitrobutane	4NO	87.75	17.30	4	
2,4,5-trimethylthiazole	tMthz	66.54	16.39	13	
2-acetylthiophene	acthz	65.20	20.17	5	
butanone	4on	64.25	14.01	4	•
methyl acetate	Mac	58.75	13.46	4	•
hexanal	6al	58.25	3.84	4	•
1-pentene	5en	57.50	5.11	4	•
3-methyl-2-butanone	M4on	56.25	10.23	4	
3-hexanone	6on	56.10	6.38	10	
3-methyl-2,4-pentanedione	Md5on	55.75	3.75	4	
acetophenone	acPhon	52.00	8.12	12	•
3-methyl-2-penten-3-one	M5enon	47.50	10.71	4	
ethyl formate	2for	47.25	15.38	4	•
cis-3-hexen-1-ol	6enol	44.55	7.58	11	
propyl butyrate	3but	42.40	5.72	10	
anisole	anisole	41.91	7.87	11	
ethyl valerate	2val	39.90	7.01	10	•
water	H2O	37.64	9.23	11	•
2-hexanol	2-6ol	37.60	8.19	10	•
3-nonanone	9on	37.40	10.29	10	
1-hexanol	1-6ol	37.00	16.22	9	•
trans-2-octen-2-one	8enon	36.50	9.23	10	
3-decanone	10on	35.78	8.31	9	
6-methyl-5-hepten-2-ol	M7enol	34.90	8.11	10	•
4-heptanone	7on	33.80	7.47	10	•
paraffin oil (solvent)	PO	33.67	8.50	15	
phenylacetaldehyde	Phacal	32.73	7.55	11	•
2,5-dimethylthiophene	dMthp	31.09	8.95	11	
1-hepten-3-ol	7enol	31.00	7.64	11	•

odorant	abbrev.	mean response (spikes/s)	s.e.m.	*n*	human odor
3-(methylthio)-propionaldehyde	Mthproal	29.00	7.12	4	
2-methyl-3-heptanone	M7on	28.00	7.92	11	•
2-heptanol	2-7ol	26.90	8.24	10	
3-heptanol	3-7ol	26.30	7.87	10	
fluoroacetone	Facon	26.00	4.00	2	
1-octanol	8ol	24.10	8.01	10	•
1-chlorohexane	6Cl	23.50	4.94	4	•
1-octen-3-ol	8enol	23.18	7.72	11	•
methoxyacetone	MOacon	19.00	5.08	4	
cis-2-hexene	Z6en	18.00	3.00	2	•
3-methyl-2-pentene	M5en	18.00	9.00	2	
1-iodohexane	6I	14.00	9.47	4	
hexylsilane	6Si	13.50	3.57	4	
1-bromohexane	6Br	13.25	9.57	4	
1-hexanethiol	6thl	13.25	6.52	4	•
pyruvic acid	pyrH	12.00	8.75	4	•
1,5-pentanediol	d5ol	8.75	7.84	4	
4-hydroxy-2-butanone	HO4on	6.25	7.70	4	
4-(methylthio)-1-butanal	Mth4ol	5.50	10.09	4	
propionic acid	proH	2.33	3.38	3	•
2-(propylamino)-ethanol	3am2ol	1.00	9.15	4	
butyryl chloride	butCl	1.00	4.34	4	
2,3-pentanedione	d5on	-2.00	6.22	4	
propionaldehyde	proal	-4.25	7.09	4	•
propionyl chloride	proCl	-5.25	5.04	4	
propionyl bromide	proBr	-8.00	4.04	4	
butanedione	d4on	-9.75	2.95	4	•
methyl pyruvate	Mpyr	-10.25	2.06	4	

Table A3.2. Human odor and related odor panel (Fig. 2.2–3) tested in *Ae. aegypti* (Rockefeller strain) and *An. gambiae* (G3 strain) cpA

All odorants diluted at 10^{-2} in paraffin oil or *water.

odorant	abbrev.	mean response (spikes/s) (*Ae. aegypti*)	s.e.m.	*n*	mean response (spikes/s) (*An. gambiae*)	s.e.m.	*n*	human odor
pyridine	prd	see above			207.90	15.91	10	•
cyclohexanone	c6on	see above			115.30	11.90	10	•
butanone	4on	see above			72.20	10.06	10	•
3-methylcyclopentanone	3-Mc5on	124.67	6.77	6	111.89	18.23	9	•
2-methylcyclopentanone	2-Mc5on	124.33	5.86	6	101.67	16.60	9	•
benzaldehyde	bzal	75.57	11.60	7	60.22	13.33	9	•
2-methylbutyraldehyde	Mbutal	72.20	7.32	5	43.67	10.12	9	•
3-methyl-3-buten-1-ol	3.M4enol	71.14	10.75	7	41.22	12.75	9	
2-methyl-1-butanol	2-M4ol	62.29	8.71	7	38.22	11.44	9	•
3-methyl-1-butanol	3-M4ol	61.14	7.84	7	29.78	10.15	9	•
1-butanol	4ol	50.43	13.32	7	73.89	7.99	9	•
2,5-dimethylpyrazine	dMprz	50.17	5.58	12	76.89	15.16	9	•
2,6-dimethylpyridine	dMprd	49.20	5.01	5	35.75	12.34	9	
1-pentanol	5ol	48.43	7.40	7	35.00	7.66	9	•
4-methyl-3-penten-2-one	M5enon	44.71	6.73	7	36.56	9.34	9	•
3-methyl-2-buten-1-ol	2.M4enol	43.67	7.37	6	27.44	9.37	9	•
2,3,5-trimethylpyrazine	tMprz	38.60	9.79	5	26.33	6.76	9	•
3-hexanol	3-6ol	36.43	8.85	7	29.80	8.86	10	•
3-acetoxy-2-butanone	ac4on	32.67	3.62	6	36.67	10.27	9	•
2-ethyl-3,5(or 6)-dimethylpyrazine	2dMprz	14.69	5.34	13	17.33	6.80	9	•
linalool	lnl	18.00	2.31	3				

odorant	abbrev.	mean response (spikes/s) (*Ae. aegypti*)	s.e.m.	*n*	mean response (spikes/s) (*An. gambiae*)	s.e.m.	*n*	human odor
octanal	8al	16.67	5.16	6	7.89	4.60	9	•
2,3-dimethyl-2-cyclopenten-1-one	dMc5enon	14.00	5.02	6	30.00	9.44	9	•
adipic acid	dhexH	10.00	6.03	3				•
5-nonanone	5-9on	9.50	5.21	6	9.00	4.46	9	•
3,5,5-trimethyl-1-hexanol	tM6ol	8.00	4.54	6	10.33	4.86	9	•
pyrazine	prz	6.86	2.60	7	6.67	4.24	9	•
indole	indole	6.00	4.40	7	10.56	7.70	9	•
hexanoic acid	hexH	5.86	4.53	7	1.78	4.21	9	•
naphthalene	npten	5.29	6.18	7				•
1-octanol	8ol	4.43	5.25	7	9.70	4.82	10	•
heptanoic acid	hepH	3.80	6.24	5	9.67	4.83	9	•
isovaleric acid	ivalH	3.20	4.02	5	2.44	5.62	9	•
1-decanol	10ol	2.00	3.60	6	1.89	4.49	9	•
2-furoic acid*	furH*	2.00	7.00	2	0.67	5.64	9	•
isobutyric acid	ibutH	2.00	5.21	5	4.22	3.63	9	•
2-methylhexanoic acid	MhexH	2.00	4.77	5	0.67	5.34	9	•
2-furoic acid	furH	1.67	12.57	3				•
octanoic acid	octH	1.40	4.82	5	6.89	4.40	9	•
2-nonanone	2-9on	1.33	5.35	6	3.78	3.60	9	•
acetoin	acin	0.60	2.87	10				•
paraffin oil (solvent)	PO	-0.33	2.84	18	6.27	2.75	11	
butyric acid	butH	-0.43	2.07	7	2.89	4.69	9	•
1-nonanol	9ol	-1.00	5.99	6	4.89	3.96	9	•
acetoin*	acin*	-1.00	6.03	3	2.22	5.21	9	•
2-methylnonanoic acid	MnonH	-3.80	6.02	5	6.89	4.11	9	•

odorant	abbrev.	mean response (spikes/s) (*Ae. aegypti*)	s.e.m.	*n*	mean response (spikes/s) (*An. gambiae*)	s.e.m.	*n*	human odor
trans-2-hexenoic acid	hexenH	-4.80	5.30	5	8.00	4.71	9	•
2-methyl-3,5(or 6)-diethoxypyrazine	MdEtOprz	-4.80	1.96	5				
2-methylbutyric acid	MbutH	-6.20	1.88	5	3.89	4.13	9	•
benzothiazole	bzthz	-7.80	1.96	5	10.22	6.80	9	•
2-methylheptanoic acid	MhepH	-9.60	2.73	5	5.00	6.60	9	•
2-isopropyl-3-methoxypyrazine	i3MOprz	-10.00	1.82	5				
water (solvent)	H_2O	-11.00	5.00	2	-3.00	4.58	9	•
adipic acid*	dhexH*	-13.00	0.00	2	5.00	3.08	9	•

* odorant dissolved in water

Table A3.3. Odorants identified by Bernier et al. to be behaviorally active (2003; Fig. 2.13) tested in *Ae. aegypti* (Rockefeller strain) cpA

All odorants diluted at 10^{-2} in paraffin oil.

odorant	abbrev.	mean response (spikes/s)	s.e.m.	*n*	human odor
acetone	acon	94.67	11.83	6	•
dichloromethane	dClM	86.83	4.83	6	
dimethyl disulfide	dMdS	79.33	3.86	6	•
paraffin oil	PO	22.75	5.05	8	
methanol	Mol	13.17	2.48	6	•

Appendix 4. Sequences of cloned *Ae. aegypti* gustatory receptors Gr1,2,3

>AaGr1
ATGATTCACAGCCAGATGGAAGATTCGCAGTACCAGATTCGGCAGCAGATTTTGAACCCGAACCAACGCCA
ACAGCTGGAGGATAACCGCCGCATCAAAGAGCAGATGCAGCAGTTGCAAAGAGATGATGCATCGCCAAGCC
ACATGTACATCCGCAAGTTGGAGTTCCAGGCCGATGTGAACCTTCTGGACAAACACGATTCTTTCTACCAC
ACCACCAAAAGCCTGTTGGTGCTATTTCAAATAATGGGAGTCATGCCAATCGTCAGAAGTCCCAAAGGTGT
CAACATGCCCCGAACCACATTCACCTGGTGCTCGAAAGCCTTCATCTGGGCGTACTTTATCTACGCTTGCG
AAACGGTTCTTGTTGTCCTAGTAGCCAAAGAGCGCATCAAACGTTTCATTTCGACCAGCGACAAACGATTC
GACGAAGTGATCTACAATATCATTTTTATGAGCCTTTTGGTTCCGCATTTCCTCCTTCCGGTGGCATCCTG
GCGCAATGGATCGGAGGTGGCCAAGTTCAAGAACATGTGGACCGACTACCAGTATAAGTACCTCATGGTCA
CCGGAAAGCCGATCGTCTTTCCGAAGCTCTATCCGATCACGTGGGTTTTGTGTGTGGTTTCGTGGGCTGTT
AGTTTTGTgATCATCATGTCCCAATAtTATTTGCAACCGGATTTCCAACTGACGCAtACATTTGCCTATTA
TCACATTTTGGCGATGTTGAACGGGTTTTGTAGTTTATGGTTCGTCAACTGTACAGCCTTTGGGACGGCAA
GCAAGGCATTTGCGCAGGAACTGTCGAATATATTTtGCCACtGAGCAACCTGCCGACAAACTGACCGAATAT
CGTCATCTGTGGGTTGATCTTAGCCATATGATGCAGCAATTGGGAAAAGCGTACTCGAACATGTATGGCAT
CTATTGTTTGGTGATTTTCTTCACCACAATTATCGCCACCTATGGGGCGCTGAGCGAAATCATCGAGCACG
GAGCGACCTATAAGGAGGTCGGTTTATTCGTCATTGTGTTCTACTGCATGGGTCTGCTGTTCATCATCTGC
AACGAGGCCCATCACGCCTCCAGAAGGGTTGGATTGAATTTCCAAGAACGGCTACTCAACGTGAACCTAAC
GGCGGTGGACAAGGCGACGCAGAAGGAGGTGGAAATGTTTCTGGTGGCCATCGATAAAAATCCACCGACGA
TGAATCTGGACGGGTATGCGAACATCAATCGCGGATTGATTACATCGAATATATCGTTCATGGCTACCTAT
CTGGTGGTGCTGATGCAGTTCAAGTTGACCCTGTTGCGACAGAGTGCCCGAAAAGCTCTCATCCCAGCTCT
GCGAGCGAATCTAACTAAGCTGAAGGAGAACTAG

N Point mutation from AaeL published sequence
t Point mutation polymorphic in Orlando lab strain (see below)
NNN Sense mutation reflected in aa sequence
TAG Stop codon

Detected polymorphisms and comparison with reference sequence AaeL:

bp	minor allele/Major allele	AaeL	SNP type
648	g/C	G	Silent mutation: V.
666	t/C	T	Silent mutation: Y.
696	t/C	T	Silent mutation: H.
816	t/A	A	Sense mutation: L272F.
822	t/C	C	Silent mutation: T.

>AaGr1 predicted protein sequence
MIHSQMEDSQYQIRQQILNPNQRQQLEDNRRIKEQMQQLQRDDASPSHMYIRKLEFQADVNLLDKHDSFYH
TTKSLLVLFQIMGVMPIVRSPKGVNMPRTTFTWCSKAFIWAYFIYACETVLVVLVAKERIKRFISTSDKRF
DEVIYNIIFMSLLVPHFLLPVASWRNGSEVAKFKNMWTDYQYKYLMVTGKPIVFPKLYPITWVLCVVSWAV
SFVIIMSQYYLQPDFQLTHTFAYYHILAMLNGFCSLWFVNCTAFGTASKAFAQELSNIFATEQPADKLTEY
RHLWVDLSHMMQQLGKAYSNMYGIYCLVIFFTTIIATYGALSEIIEHGATYKEVGLFVIVFYCMGLLFIIC
NEAHHASRRVGLNFQERLLNVNLTAVDKATQKEVEMFLVAIDKNPPTMNLDGYANINRGLITSNISFMATY
LVVLMQFKLTLLRQSARKALIPALRANLTKLKEN-

Sense mutations:
F102C
L272F

>AaGr2
ATGGTCATCAAAGACAGTGAGTTCGAGGATTCGCTCAACTACGCGCTGCTTCGCGGCGATATGGGCACAAC
CTGGGATATCAACAAAGATGAACGCATGATGAACGGGACTCTGGATCCGGAGTTGATTCAGCGAGCCAAGG
AACGAGCTATCCGGGCGCAGTTGAATTCGGCAGATGGAGATACCTGTGAGCTTCACGACCAGTTCTACCGA
GATCATAAACTGTTGTTGGTGCTGTTTCGTGCTCTGGCTGTGATGCCTATTCTAAGATCGTCACCCGGAAG
AATCACCTTCGACTGGAGATCCTGGGCCTCGATCTACGCGTACTGCTTCTACGTTGTTAGCACTGTGATTG
TACTCATTGTGGGATACGAACGGTTTAAGATTCTACAAGATACCAAGAAATTCGACGAATACATCTACGGA
GTTCTGTTCATAATTTTCTTGGTACCACACTTCTGGATTCCATTCGTAGGATGGGGAGTAGCAAAGCATGT
CGCTGTCTACAAAACGATGTGGGGAGCGTTTCAAGTTCGATACTACCGCGTTACGGGAACCAACCTCCAAT
TCCCGCATCTCAAAATCCTCATCGTCATGTTCTCGATTGGCTGCTTGGTCTGCGCCATAGTGTTTCTCTTA
TCGCTCAGTTTCCTCCTGGAAGGATTCGCGTTGTGGCACACTTCCGCTTACTATCATATCATTACCATGCT
GAACATGAACAGTGCTTTGTGGTACATCAACTGTCGTGGAATACGGGTAGCCTCGTCCAGTTTGTCTGACC
GTTTCCGCAAGGACGTTGCCATCGAATGTACCGCGGCAATGATTTCGCAGTACCGCTTCCTCTGGTTGAAC
CTCAGCGAGCTGCTGCAAGCCCTGGGAAACGCCTACGCTAGAACCTATTCCACGTATTGTCTTTTTATGTT
CGCTAACATTACGATTGCCATCTACGGTGCTCTGTCGGAAGTAATTGACCACGGGTTCGGGTTTTCGTTTA
AGGAAATTGGATTGATCGTGGACACGGTCTACTGTTCGACCTTGCTGTTCATTTTTTGCGACTGTTCCCAC
AATGCTACACTGCAAGTGGCCCAGGGAGTTCAGGATACGCTACTTGGTATCAATTTGTTGAAGGTGGGCCA
TCCAACTCAGAAGGAGATCGATCTGTTCATACAGGCAATCGAGATGAATCCGGCTATCGTGAGCCTGAAGG
GTTATGCCGAAGTGAACCGGGAGTTGCTAACGGCGAGCATTGCCACCATTGCGATCTACCTGGTTGTATTG
CTGCAGTTTAAGCTATCGTTGATTTCGCAACAAATGCCGATTGAATTGATGGAAATCAAGCACAGTCATAA
GGGATAG

Alternate isoform 5′ end (sequenced from gDNA):
ATGACAGTAATTGCTATAAAGGTTGTGGAGAAAGCGAATTACCGAACAGTTGATTATCACGTCCTGCGAAA
GAAA

>AaGr2 predicted protein sequence
MVIKDSEFEDSLNYALLRGDMGTTWDINKDERMMNGTLDPELIQRAKERAIRAQLNSADGDTCELHDQFYR
DHKLLLVLFRALAVMPILRSSPGRITFDWRSWASIYAYCFYVVSTVIVLIVGYERLKILQDTKKFDEYIYG
VLFIIFLVPHFWIPFVGWGVAKHVAVYKTMWGAFQVRYYRVTGTNLQFPHLKILIVMFSIGCLVCAIVFLL
SLSFLLEGFALWHTSAYYHIITMLNMNSALWYINCRGIRVASSSLSDRFRKDVAIECTAAMISQYRFLWLN
LSELLQALGNAYARTYSTYCLFMFANITIAIYGALSEVIDHGFGFSFKEIGLIVDTVYCSTLLFIFCDCSH
NATLQVAQGVQDTLLGINLLKVDHPTQKEIDLFIQAIEMNPAIVSLKGYAEVNRELLTASIATIAIYLVVL
LQFKLSLISQQMPIELMEIKHSHKG-

Sense mutations:
Lacks N terminus: MTVIAIKVVEKANYRTVDYHVLRKK

>AaGr3
ATGAATCTCAACCAAGACCCTATTCAGTACATCAATTTGAATAACAATGCTCGAACGGTTTTTCTGGACGT
GAAACCAATTTACAACGAAGAGAAGCGTAAAGTTTCTAATGGATTTAACAATCGCATTGGATTTCCGCCAA
TCTCTTCGAGGAGAGTGTTCGGTTTGGAAAGTGACTTCAACACGCGATCGGATATAGTTTACGGCACCACG
AAGCCAATCTACAACGTTCTACGGATGCTGGGAGTGTTTCCTTTCTCGAGACCTTCACCCGGAGTGACATT
ATTTGCCTGTGCGAGCCCTGCGATGGCATATTGCAGTGTGTTTTTCGTGACGCTTATGGCTTACGTCATCT
ACATAACCATCCTCCGGGTCCATATTGTCCGCACACTGGAAGGCCGCTTCGAAGAGGCCGTCATCGCTTAT
CTCTTCATCGTTAACATCCTGCCGGTTCTGATCATTCCGTTGATGTGGTACGAAACTCGTAAGGTCTCCAG
TTTGCTCAATCAATGGGTCGACTTCGAGGCAATCTATCGTAAAACTGCGGGCAGAGAACTGGAGCTGTCAT
TCAGAACCAAAGCCCTACTGATTGCTATTTTACTGCCGGTTCTGTCTTGTCTGGCAGTCATCATAACCCAC
GTGACAATGGTTGAATTCCAGCTGGTTCAGGTCATCCCGTATTGCATTCTGGACACGCTAACCTATATGAT
GGGAGGTTATTGGTATATGACCTGCGAAACTCTCAGTATAACTGCCAACATTCTGGCGGAGGATTTCCAAA
GGGCTTTGAGGCACGTTGGACCAGCAGCTATGGTATCCGAATACCGCTCGCTATGGCTGCGATTGAGTAAG
CTTGCTCGAGAAACGGGATCATCCACGTGTTACACCTTCACCTTTCTGTGTCTATATCTCTTCTTCATCAT
CACTCTTTCGATCTACGGCCTGATGTCGCAAATTTCCGAAGGTTTCGGCATCAAAGACATCGGCCTGGCAG
TGACCGCCTTCTGCAGCGTTGGGTTACTCTTCTTCATATGCGACGAAGCTCACTACGCGTCGTTCAACGTT
CGGACCAAATTCCAGAAGAAGTTGTTGATGGCAGAGCTCAGTTGGATGAACTCGGATGCACAAACCGAAAT
CAACATGTTTCTGAGGGCAACCGAGATGAATCCTTCGAGCATCAACTTGGGCGGGTTTTTCGACGTGAACC
GGACGCTGTTCAAATCGCTTTTGGCAACGATGGTGACCTATTTGGTGGTGTTGCTACAGTTCCAAATCAGC
ATACCAGACGACTCTAGCATGTTAGTGATGCATAATATGACGGGTTCATATCGCGAGTAG

N Point mutation from AaeL published sequence

>AaGr3 predicted protein sequence
MNLNQDPIQYINLNNNARTVFLDVKPIYNEEKRKVSNGFNNRIGFPPISSRRVFGLESDFNTRSDIVYGTT
KPIYNVLRMLGVFPFSRPSPGVTLFACASPAMAYCSVFFVTLMAYVIYITILRVHIVRTLEGRFEEAVIAY
LFIVNILPVLIIPLMWYETRKVSSLLNQWVDFEAIYRKTAGRELELSFRTKALLIAILLPVLSCLAVIITH
VTMVEFQLVQVIPYCILDTLTYMMGGYWYMTCETLSITANILAEDFQRALRHVGPAAMVSEYRSLWLRLSK
LARETGSSTCYTFTFLCLYLFFIITLSIYGLMSQISEGFGIKDIGLAVTAFCSVGLLFFICDEAHYASFNV
RTKFQKKLLMAELSWMNSDAQTEINMFLRATEMNPSSINLGGFFDVNRTLFKSLLATMVTYLVVLLQFQIS
IPDDSSMLVMHNMTGSYRE-

Sense mutations:
E59D
G107S
L109F

Appendix 5. Genotypes and sources of *Drosophila* used in Chapter 3.

construct name	genotype	method, creator
wild type	*wCS*	
AaGr1 on II	*UAS–AaGr1A16*	ΦC31 injection in attP40 site
AaGr1 on III	*UAS–AaGr1C49*	ΦC31 injection in VK00027 site
AaGr2 on II	*UAS–AaGr2A10*	ΦC31 injection in attP40 site
AaGr2 on III	*UAS–AaGr2C45*	ΦC31 injection in VK00027 site
AaGr3 on II	*UAS–AaGr3A2*	ΦC31 injection in attP40 site
AaGr3 on III	*UAS–AaGr3C46*	ΦC31 injection in VK00027 site
Ag22	*UAS–AgGr22*	P element insertion on II, I. Coutinho-Abreu
Ag23	*UAS–AgGr23*	ΦC31 injection in attP40 site, I. Coutinho-Abreu
Ag24	*UAS–AgGr24*	P element insertion on II, I. Coutinho-Abreu
Pp22	*UAS–PpGr22*	P element insertion on II, I. Coutinho-Abreu
Pp24	*UAS–PpGr24*	P element insertion on II, I. Coutinho-Abreu
ΔGr21a	$Gr21a^{3}$	CRISPR deletion, S. Perry
ΔGr63a		Bloomington *Drosophila* Stock Center 9941
G4	*Gr63a–GAL4*	Bloomington *Drosophila* Stock Center 9942
ΔGr63a,G4		Recombinant, E. G. Freeman

Iliano Coutinho-Abreu, Sarah Perry: Ray laboratory (UCR). Erica G. Freeman: Dahanukar laboratory (UCR).

Fly genotypes used for empty neuron experiments (Table 3.1)

name	full genotype
−;−	*ΔGr21a; ΔGr63a*
−;G4	*ΔGr21a; ΔGr63a/ΔGr63a,G4*
1;G4	*ΔGr21a,AaGr1/ΔGr21a; ΔGr63a/ΔGr63a,G4*
2;G4	*ΔGr21a,AaGr2/ΔGr21a; ΔGr63a/ΔGr63a,G4*
3;G4	*ΔGr21a,AaGr3/ΔGr21a; ΔGr63a/ΔGr63a,G4*
1,2;G4	*ΔGr21a,AaGr1/ΔGr21a,AaGr2; ΔGr63a/ΔGr63a,G4*
1,3;G4	*ΔGr21a,AaGr1/ΔGr21a,AaGr3; ΔGr63a/ΔGr63a,G4*
2,3;G4	*ΔGr21a,AaGr2/ΔGr21a,AaGr3; ΔGr63a/ΔGr63a,G4*
1,2,3;G4	*ΔGr21a,AaGr1/ΔGr21a,AaGr2; ΔGr63a,AaGr3/ΔGr63a,G4*
	ΔGr21a,AaGr1/ΔGr21a,AaGr3; ΔGr63a,AaGr2/ΔGr63a,G4
	ΔGr21a,AaGr2/ΔGr21a,AaGr3; ΔGr63a,AaGr1/ΔGr63a,G4
1,2,3;−	*ΔGr21a,AaGr1/ΔGr21a,AaGr2; ΔGr63a,AaGr3/ΔGr63a*
	ΔGr21a,AaGr1/ΔGr21a,AaGr3; ΔGr63a,AaGr2/ΔGr63a
	ΔGr21a,AaGr2/ΔGr21a,AaGr3; ΔGr63a,AaGr1/ΔGr63a

Fly genotypes used for full neuron/gain of function experiments (Table 3.2)

name	**full genotype**
1;G4	*AaGr1/+; +/G4*
2;G4	*AaGr2/+; +/G4*
3;G4	*AaGr3/+; +/G4*
1,2;G4	*AaGr1/AaGr2; +/G4*
1,3;G4	*AaGr1/AaGr3; +/G4*
2,3;G4	*AaGr2/AaGr3; +/G4*
1,2,3;G4	*AaGr1/AaGr2; AaGr3/G4*
	AaGr1/AaGr3; AaGr2/G4
	AaGr2/AaGr3; AaGr1/G4
1,2,3;+	*AaGr1/AaGr2; AaGr3/+*
	AaGr1/AaGr3; AaGr2/+
	AaGr2/AaGr3; AaGr1/+

Fly genotypes used for hybrid receptor experiments (Table 3.3)

name	**full genotype**
Δ21a,Aa1;G4	*ΔGr21a,AaGr1; G4*
Δ21a,Aa2;G4	*ΔGr21a,AaGr2; G4*
Δ21a,Aa1/2;G4	*ΔGr21a,AaGr1/ΔGr21a,AaGr2; G4*
Aa3;Δ63a,G4	*AaGr3; ΔGr63a,G4*
Δ21a,Ag22;G4	*ΔGr21a,AgGr22; G4*
Δ21a,Ag23;G4	*ΔGr21a,AgGr23; G4*
Δ21a,Ag22/23;G4	*ΔGr21a,AgGr22/ΔGr21a,AgGr23; G4*
Ag24;Δ63a,G4	*AgGr24; ΔGr63a,G4*
Δ21a,Pp22;G4	*ΔGr21a,PpGr22; G4*
Pp24;Δ63a,G4	*PpGr24; ΔGr63a,G4*

Nb: The GRs used in this study have different names in *Anopheles* and *Phlebotomus* than in other insects. Gr22 from those species is orthologous with AaGr1, Gr23 is orthologous with AaGr2, and Gr24 is orthologous with AaGr3.

Appendix 6. Responses to CO_2 in *Aedes aegypti aegypti* and *Ae. aegypti formosus*.

Most of this dissertation has discussed host-seeking behavior of *Aedes aegypti aegypti*, which is highly anthropophilic and globally distributed. There is another subspecies, *Aedes aegypti formosus* (Walker, 1848), which is primarily sylvan and zoophilic. These two subspecies diverged recently, probably in West Africa (Sylla et al. 2009). They interbreed freely in the laboratory, but where their wild ranges overlap gene flow occurs slowly because of distinct habitat preferences and reduced rates of genetic recombination due to chromosomal inversions (Bernhardt et al. 2009).

Exhaled carbon dioxide (CO_2) is a general host cue used by many species of mosquito, including *Aedes aegypti* (reviewed in Chapter 1). When a host-seeking mosquito contacts an odor plume of CO_2, the odor triggers upwind flight that continues until she loses the CO_2 plume or locates the source. Contact with CO_2 also sensitizes *Ae. aegypti* to host odor (Dekker et al. 2005).

Methods

In this experiment, flight behaviors in response to CO_2 were investigated in five strains of *Ae. aegypti* recently collected from the wild, representing both subspecies, as well as a well-studied laboratory strain for comparison. The five wild strains were provided by W. C. Black IV (Colorado State University) from field collections in Senegal. Four of them were selected to include individuals of one or the other subspecies, and one mixed strain allows interbreeding (as indicated in Table A6.1).

Behavior tests were conducted in a Plexiglas wind tunnel in the Cardé laboratory at UCR. This wind tunnel measures 50 cm × 50 cm × 150 cm (length) and is described in

detail in Dekker et al. (2005) and Lacey et al. (2014). Air was pushed into the tunnel from outside the building by an in-line duct fan, steam humidified, charcoal filtered, and passed through a honeycomb laminizer at a controlled rate of 30 cm/s at ~27° and 70% relative humidity. A turbulent plume of CO_2 was generated by pumping 1% CO_2 through a perforated glass ring at the upwind end of the wind tunnel.

	Aedes aegypti aegypti		***Aedes aegypti formosus***		**mixed**	**lab strain**
strain name	AaaS	AaaB	AafS	AafB	AaMix	AaR (Rockefeller)
collection location	Kaolack, Senegal	Bignona, Senegal	Sediou, Senegal	Bignona, Senegal	PK-10, Senegal	in culture >84 years

Table A6.1. Test strains of *Ae. aegypti* mosquitoes

Two to eight–day-old female mosquitoes were isolated and starved between 4 and 24 hours before testing. Each mosquito was individually released at the downwind end of the tunnel and behavior was video recorded for up to 5 min. Videos of mosquito behavior were analyzed by C. Umeda. Whether and when mosquitoes left the release cage (Activation), flew at least halfway up the wind tunnel (Upwind flight), or reached the CO_2 emitter (Source finding) were recorded relative to the start of the assay. Assays were conducted in a randomized complete block design, but this did not have a significant effect, with one exception. Mosquitoes in one block of tests conducted relatively late in the day were less likely to fly upwind than mosquitoes from the other 7 blocks (one-margin fixed 2×8 X^2 test, $p < 0.05$), so results from that block were not considered in statistical analyses.

Results

All but one mosquito activated within 5 min of release, and 42 out of 48 tested mosquitoes flew upwind. All mosquitoes that flew upwind also reached the CO_2 emitter.

There were no significant differences among the six strains in when they took off, reached the halfway point of the wind tunnel, or reached the emitter (Fig. A6.1, one-way ANOVA of log transforms of times recorded for each behavior, $p > 0.05$). The effect of strain on activation time was marginally significant ($p = 0.07$), probably due to the trend of the laboratory strain (AaR) to activate more quickly, so it is possible that the difference in activation time may reach significance with additional replicates.

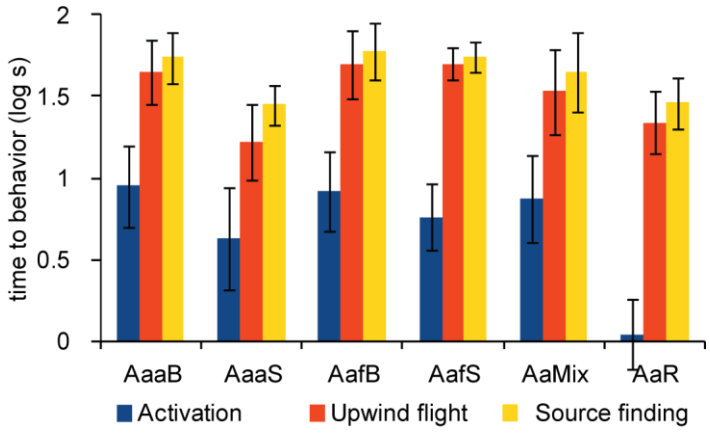

Figure A6.1. Summary of CO_2 flight behavior across *Ae. aegypti* subspecies.
Means of log transformed measurements of time until observed behaviors across wild-caught strains and a laboratory strain (AaR). $n = 5–8$. Error bars are s.e.m.

Discussion

No differences were detected between *Ae. aegypti aegypti* and *Ae. aegypti formosus* in behavior towards a CO_2 stimulus. Even though mosquitoes from the laboratory strain tended to leave the release cage earlier in the assay, this was not reflected in faster time to fly upwind or reach the CO_2 source. There were no clear differences in CO_2-evoked behavior between any of the recently colonized strains, indicating this behavior cannot be used to distinguish the two *Ae. aegypti* subspecies. This is in contrast with their preference for humans over a guinea pig, which has been linked to a specific odorant receptor separate from the CO_2 detection pathway (McBride et al. 2014).