Odor Coding of Social Behavior in Eusocial Ants

By

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I dedicate this dissertation to my family. To my parents, Duane and Cheryl Ferguson, for their unyielding support and unconditional love. To my sister, Lisa Beckman, for her ecouragement and solidarity. And to my niece, Brynlie Beckman, for her friendship.

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Introduction

Olfaction arguably represents one of the most important sensory modalities for insects. This is especially true for Hymenoptera which engage in a variety of complex olfactory-mediated social behaviors that distinguish them from other insect orders such as Diptera. Indeed, while model insects such as Drosophila melanogaster have played fundamental roles in advancing our understanding of the molecular, cellular, and organismal components of insect olfaction in general, the extension of these studies to a Hymenopteran model species has provided an opportunity to address a broader range of questions related to insect pheromone biology, especially as it pertains to social behavior and evolution.

Hymenoptera

Hymenoptera is an evolutionarily successful and globally pervasive order of holometabolous insects which includes ants, bees, and wasps (suborder Apocrita) as well as the more primitive sawflies (suborder Symphyta). It has been suggested that Hymenoptera may represent the most species-rich insect order (Forbes et al. 2018) with ants alone comprising, on average, more than 15% of all terrestrial biomass (Schultz 2000). This diverse order of insects has collectively captured the attention of scientists across a broad range of disciplines due to their unique social structures and their significance as crop plant pollinators, agricultural pests, seed dispersers, and drivers of soil turnover (Hölldobler and Wilson 2009).

The prevalence of at least some of these insects may be attributed to their eusocial organization, defined by: 1) a reproductive division of labor, 2) overlapping generations, and 3) cooperative brood care. As a result of this unique social architecture, there are several characteristics that distinguish eusocial colonies from other solitary insect orders. Perhaps the most notable characteristic in this regard is that sex-determination in Hymenoptera occurs through a haplodiploid system such as arrhenotoky. In other words, sex is determined by the number of copies of each chromosome possessed by an individual. Colonies are typically founded by one or

[*] This chapter was published in 2020 in the textbook *Insect Pheromone Biochemistry and Molecular Biology (2nd Ed.)*, pages 507-546, with myself as first author. Anandasankar Ray was a co-author. L.J. Zwiebel was senior author.

more reproductive female queens after engaging in a mating flight. Throughout her lifetime, a queen may lay fertilized diploid eggs which develop into effectively sterile female workers or reproductive virgin queen daughters. Alternatively, unfertilized haploid eggs will develop into reproductive males.

There are, however, notable exceptions to this rigid reproductive framework. In the ponerine ant *Harpegnathos saltator*, for example, after the death of the queen, workers will engage in a ritualized display known as dueling (Peeters, Liebig, and Hölldobler 2000; Peeters and Hölldobler 1995). The "winner" of these aggressive bouts will become the new reproductive in the colony, referred to as a gamergate. Another example is the queenless clonal raider ant *Ooceraea biroi*. All members of the raider ant colony are capable of clonal reproduction through thelytokous parthenogenesis (Tsuji and Yamauchi 1995). In this system, workers within a colony alternate between a non-reproductive foraging phase during which food is provisioned for the colony and a reproductive phase during which unfertilized eggs develop into diploid females (Ravary and Jaisson 2002). Importantly, these unique features of the reproductive system have facilitated the application of genetic engineering technologies to move towards genetic models which would otherwise be challenging in light of the long generation time of colonies and the sterility of the worker caste (Yan et al. 2017; Trible et al. 2017).

Another compelling element of the biology of many Hymenopteran species is that female offspring are, on average, 75% genetically identical. This is because workers ubiquitously inherit the same paternal chromosome from their haploid father and one of the two chromosomes from their diploid mother. Yet despite high levels of genetic similarity, there is a profound lack of homogeneity within members of a single colony in terms of age, morphology, and behavior (Hölldobler and Wilson 1990; Wheeler 1986). Female queens, some of which may live for nearly 30 years in some species such as *L. niger* (Keller 1998; Keller and Genoud 1997; Hölldobler and Wilson 1990), are much larger than their offspring and devote much of their time to egg laying. Workers may only live for a few weeks or months and carry out the remainder tasks within the colony such as brood care, nest maintenance, and foraging (Hölldobler and Wilson 1990; Page and Peng 2001). In certain species, workers may be further differentiated into morphological castes which perform specialized tasks within the colony (Hölldobler and Wilson 1990). Males, on the other hand, typically only live for a few weeks and die shortly after mating (Wilson 1971;

Hölldobler and Wilson 1990). These differences are reflected in the physiology of each individual with important implications for the structure and function of the olfactory system.

Considerable attention has also been given to more basic and fundamental scientific questions regarding Hymenopteran social biology, neuroethology, sensory systems, and evolution. Charles Darwin himself was particularly troubled by observations made of eusocial insects. In his seminal book the *Origin of Species*, Darwin laments that sterile female workers from ants and other eusocial insects, "at first appeared to me insuperable, and actually fatal to my whole theory… for these neuters often differ widely in instinct and in structure from both the males and fertile females, and yet, from being sterile, they cannot propagate their kind" (Darwin 1859). While subsequent studies by evolutionary scientists such as W.D Hamilton have since provided a conceptual framework for understanding the evolution of reproductive altruism through kin selection (Hamilton 1963, 1964), genome sequencing has paved the way to examine this biology and ask new questions about the origins of eusociality. For example, what are the molecular requirements of communicating social information and maintaining complex colonial lifestyles with reproductive hierarchies? And what are the selective pressures that might drive these organisms towards eusociality?

Hymenoptera Pheromone Biology

Beyond the basic tenets of eusociality, these insects engage in a range of complex social behaviors. Importantly, many of these behaviors are largely thought to be mediated by the production and detection of specific chemical cues (Endler et al. 2004; Wagner et al. 1998; Lang and Menzel 2011; Wagner et al. 2000; Greene and Gordon 2003; Heimken, Aumeier, and Kirchner 2009). Indeed, Hymenoptera has a storied history of chemical ecological studies detailing the relationships between pheromones and a wide range of important behaviors preceding more recent advances in molecular techniques. While not all social behaviors depend solely on chemosensory or, more specifically, olfactory signaling, it is clear that the chemical-based communication of social information represents a vital aspect of eusociality.

Post eclosion, young sterile female workers tend to the queen's offspring as nurses. Specious reproductives, such as workers with developed ovaries, may also produce offspring. While oftentimes these workers begin egg laying in queenless colony conditions in the absence of pheromonal or behavioral suppression of ovary development, a small proportion of workers in

queenright colonies may also have developed ovaries and contribute to the production of males within a colony (Kuszewska et al. 2018; Bourke 1988; Jay 1968). Yet in these rare or otherwise exceptional cases, workers are able to distinguish between queen- and worker-laid eggs based on distinct chemical signatures found on the surface of the eggs (Endler et al. 2004; Helantera and Sundstrom 2007). Both ants and honeybees preferentially destroy worker-laid eggs relative to queen-laid eggs (Endler et al. 2004; Ratnieks and Visscher 1989). Furthermore, the transition from a worker to a reproductive is typically accompanied by changes in the chemical profile of the cuticular that signal fertility (Liebig et al. 2000; Gobin, Billen, and Peeters 1999; Kikuta and Tsuji 1999; Liebig, Peeters, and Holldobler 1999). These cues are also used by workers to actively aggress specious reproductive and suppress the activation of their ovaries (Liebig, Peeters, and Holldobler 1999; Kikuta and Tsuji 1999). These policing behaviors ensure a stable division of labor within the colony (Ratnieks 1988).

As workers age, they will begin transitioning to other tasks necessary for colony maintenance, such as structuring and cleaning the nest (Seeley 1982a; Sommeijer 1984; Wilson 1976; Mikheyev and Linksvayer 2015; Jeanne, Williams, and Yandell 1992). Perhaps the most notorious chemical cues in this regard are the so-called life and death pheromones. As workers die within a colony, they decompose and begin emitting chemicals such as oleic acid that release necrophoric behaviors (Visscher 1983; Gordon 1983; McAfee et al. 2018). Individuals that carry this chemical mark of death are disposed of as refuse and carried out of the colony. Other studies have demonstrated that the absence of certain chemical signatures associated with life, such as dolichodial and iridomyrmecin in the ant *L. humile*, can also elicit the rapid removal of dead workers from within a colony (Choe, Millar, and Rust 2009). Interestingly, the response to these odor cues is modulated by the behavioral status of the colony. When workers of the harvester ant *Pogonomyrmex badius* were engaged in cleaning behaviors, oleic acid was perceived as a death pheromone and paper treated with this compound was removed from the nest. However, oleic acid is also commonly found on the seeds collected by this species. When workers were engaged in foraging behavior, papers treated with oleic acid were instead carried into the nest (Gordon 1983).

The oldest workers routinely leave the nest in order to gather food for the colony as foragers (Seeley 1982a; Sommeijer 1984; Wilson 1976; Mikheyev and Linksvayer 2015; Jeanne, Williams, and Yandell 1992). Foraging is a complex process and involves the integration of information through several different sensory systems including chemical cues from a variety of exogenous

and colony-related sources. While the spatial-temporal dynamics of the honeybee dance have been well-described (von Frisch 1967), these dances are also accompanied by the release of a discrete set of hydrocarbons that modulate foraging activity (Thom et al. 2007; Gilley, Kuzora, and Thom 2012; Gilley 2014). In the harvester ant *Pogonomyrmex barbatus*, foraging is also regulated through a multimodal process. Successful foragers returning to the nest communicate two distinct cues: a forager-associated hydrocarbon profile and the food odor. However, it is also the rate at which these returning foragers are encountered that elicits the recruitment of additional foragers (Greene, Pinter-Wollman, and Gordon 2013; Greene and Gordon 2003). These foragers subsequently follow species-specific trail pheromones and other cues which guide them towards the food source (Haak et al. 1996; Nieh 2004; Graham and Cheng 2009; Esch et al. 2001).

Certain ant species from the subtribe Attina have also developed agricultural systems, foraging for vegetation such as leaves which they use as a nutritional substrate for their fungal gardens (Weber 1966). Other species, such as the black garden ant *Lasius niger*, have domesticated other insects, protecting herds of aphids from predators in exchange for honeydew produced by the aphids (Banks 1958; Banks and Nixon 1958). Even larvae, which depend on the active care of their sisters, engage in social communication by influencing ovarian activation and foraging activity within the colony as well as the amount of nutrition they receive by communicating hunger status with nurses (Huang and Otis 1991; Cassill and Tschinkel 1995; Pereboom, Velthuis, and Duchateau 2003; Kaptein, Billen, and Gobin 2005; Ulrich et al. 2016).

Perhaps the most notable and well-studied class of chemical cues in Hymenoptera are the cuticular hydrocarbons (CHCs) (Bradshaw and Howse 1984; Martin and Drijfhout 2009b; Bortolotti and Costa 2014; Dani 2006; Howard and Blomquist 2005; Keeling, Plettner, and Slessor 2004). While these cuticular compounds are generally considered to act as hydrophobic barriers that prevent dessication in insects, they have been co-opted for chemical sensing and the communication of social information (Howard and Blomquist 2005). While the particular hydrocarbons present within species, which may be quite complex in terms of chain length, the presence and position of double bonds, and enantiomeric configuration, are quite similar, subtle variations in their ratio signal a variety of important information such as caste and colony membership (Nielsen et al. 1999; Tentschert, Bestmann, and Heinze 2002; van Wilgenburg et al. 2006; Foitzik et al. 2007; Martin, Helantera, and Drijfhout 2008; Wagner et al. 2000; Thomas et al. 1999; Torres, Brandt, and Tsutsui 2007; Dani 2001; Morel, Vandermeer, and Lavine 1988;

Martin and Drijfhout 2009a). This particular class of semiochemical is critically important for maintaining colony cohesion through guarding the nest and territory from non-nestmates as well as the internal organization of ants within a colony and many other behaviors within and outside the nest heyev(Seeley 1982a; Sommeijer 1984; Wilson 1976; Mikheyev and Linksvayer 2015; Jeanne, Williams, and Yandell 1992; Heyman et al. 2017). The most striking example of colony cohesion in this context comes from invasive ant species which may form so-called "super-colonies" spanning large territorial regions; within which, there are low levels of intraspecific aggression (Holway et al. 2002). In the Argentine ant *L. humile*, a massive transcontinental supercolony has been discovered which spans thousands of kilometers across North America, Europe, and Asia (Tsutsui et al. 2000; Giraud, Pedersen, and Keller 2002; Sunamura et al. 2007; Sunamura, Hatsumi, et al. 2009; Sunamura, Espadaler, et al. 2009). Members of this super-colony possess similar CHC labels and exhibit low levels of aggression towards one another relative to Argentine ants from other, unrelated super-colonies (Sunamura, Espadaler, et al. 2009).

While the primary importance of hydrocarbon detection in mediating social interactions within and between colonies has long been recognized (Hölldobler and Wilson 1990), recent studies have also suggested that the external microbiome may similarly influence aggression between non-nestmates insofar as ants treated with a microbial culture are rejected from their nest by their nestmates but those treated with the topical antibiotic rifampin (1%) are not rejected (Dosmann, Bahet, and Gordon 2016). Behavioral task groups such as nurses and foragers also possess different CHC profiles (Kather, Drijfhout, and Martin 2011; Martin and Drijfhout 2009a; Smith and Taylor 1990; Bonavitacougourdan, Clement, and Lange 1993; Kaib et al. 2000; Wagner et al. 1998), and the recognition of task-specific cues is important for regulating certain social behaviors (Greene and Gordon 2003). For example, the duration and orientation of the waggle dance of foraging honeybees encodes spatial information such as the distance and direction to a food source, respectively (von Frisch 1967). However, foragers have also been shown to release a discrete subset of alkanes (tricosane and pentacosane) and alkenes (Z-9-tricosene and Z-9-pentacosene) which effectively increase the hive's foraging activity (Thom et al. 2007; Gilley, Kuzora, and Thom 2012; Gilley 2014). Changes in reproductive status also alters individual odor profiles, and the detection of fertile worker hydrocarbons leads to aggression and subsequent repression of ovarian development (Dietemann et al. 2003; Liebig et al. 2000; Monnin, Malosse, and Peeters 1998; Cuvillier-Hot et al. 2001; Peeters, Monnin, and Malosse 1999; Sledge, Boscaro,

and Turillazzi 2001; Hannonen et al. 2002; Heinze, Stengl, and Sledge 2002). While this brief summary of hydrocarbons is decidedly non-exhaustive, and entire textbooks can be, and indeed have been, dedicated to discussions about the importance of insect hydrocarbons (Blomquist and Bagneres 2010), suffice it to say this class of semiochemicals plays an especially critical role in Hymenopteran biology. However, the precise role of the olfactory system and the genomic and molecular requirements of pheromone detection in Hymenoptera have only recently been made possible, and there is still much to be discovered.

The Hymenopteran Olfactory

While the fundamental structure and organization of the peripheral and central olfactory system is conserved across insect orders, the Hymenopteran olfactory system has notable differences relative to other insects such as members of the well-studied order of Diptera. Of particular interest is the complex peripheral olfactory system, with a large number of diverse hair-like structures known as sensilla that decorate sensory appendages housing potentially dozens of chemoreceptor neurons that are responsible for the initial signal transduction processes. Beyond these diverse set of peripheral sensors, hymenopteran chemosensory information is integrated and processed in the antennal lobe, which displays significant differences in development and neuroanatomy relative to *Drosophila*, and the mushroom body and lateral horn of the protocerebrum of the insect brain.

Peripheral Olfactory Sensory System

As with other insects, Hymenopteran pheromones and other semiochemicals are initially detected by a sophisticated communication system that is responsible for transducing chemical information into neuronal activity via three major classes of chemosensory receptors: odorant receptors (ORs) (Gao and Chess 1999; Clyne et al. 1999; Vosshall et al. 1999; Fox et al. 2001) and the distantly related gustatory receptors (GRs) (Clyne, Warr, and Carlson 2000) as well as the evolutionarily unrelated ionotropic receptors (IRs) (Benton et al. 2009; Abuin et al. 2011; Croset et al. 2010). These receptors can be found in dendrites of insect olfactory receptor neurons (ORNs) housed within sensilla (sensory hairs) on the antennae surface (Pask and Ray 2016). These ORNs transduce signals as action potentials when chemical stimuli pass through the sensilla pores to activate receptors on the dendrite membrane.

There are several notable differences in the structure and organization of the antenna both in terms of sex- and caste- within species as well as between Hymenoptera and other insects. The Hymenoptera antenna is comprised of three primary anatomical structure: the pedicel, the scape, and a variable number of flagellar segments which house the various sensilla. Female workers and queens typically have 10 flagellar segments whereas male drones have 11 segments (Nakanishi et al. 2009; Esslen and Kaissling 1976; Couto et al. 2017). Within a colony, ant queens have the largest antenna whereas male antenna are distinctly thin. In contrast, honeybee drones possess the largest antennae within a colony with approximately twice as much surface area per segment relative to the workers.

In addition to these gross morphological differences, there are also conspicuous differences in the organization of the sensilla along the antenna. In *Drosophila*, the distal third segment of the antenna is devoted to olfactory processing and is decorated by a total of approximately 410 sensilla (Laissue and Vosshall 2008). In contrast, Hymenoptera sensilla are on the order of thousands. Honeybee drones, for example, have an astonishing 20,000 sensilla per flagellum with workers possessing around 6,000 (Esslen and Kaissling 1976). There are also sex- and caste- specific differences in the chemosensory ultrastructure of ants. Queens appears to have the most sensilla. In *C. japonicus*, this number is around 9,000 (Nakanishi et al. 2009). Males, however, have the least (6,000) with workers somewhere in the middle (7,500).

Another striking feature of the Hymenopteran antennae is the unusually high number of neurons housed within each sensory hair. While *Drosophila* olfactory sensilla have approximately 1-4 ORNs (Stocker 1994), it has been shown that the honeybee placode sensilla contains between 5-35 ORNs (Getz and Akers 1994; Kelber C 2006). These numbers are even more unusual in ants, where some trichoid sensilla have at least 50 ORNs while the hydrocarbon responsive basiconic sensilla may contain in excess of 130 ORNs (Nakanishi et al. 2009). Even in more simple systems such as *Drosophila* and *Anopheles*, the activity of one olfactory neuron may influence adjacent cells through lateral inhibition (Su et al. 2012). Such neuronal interactions have indeed been observed in honeybee olfactory sensilla (Akers and Getz 1992, 1993; Getz and Akers 1993, 1994). This large conglomeration of olfactory neurons within the Hymenopteran sensilla suggests a high level of complexity in terms of olfactory coding before these signals reach the antennal lobe.

There are also sexually dimorphic differences in the composition of sensilla subtypes, as defined by their morphology and/or function. The most prominent aspect being the absence of any

basiconic sensilla on the male antennae across many Hymenopteran species (Esslen and Kaissling 1976; Nishino et al. 2009; Nakanishi et al. 2009). However, it is worth mentioning that while these hydrocarbon responsive sensilla appear to have a similar function in wasps (Anton and Gnatzy 1998), they do not appear to be sexually dimorphic in some species. Males from both the hornet *Vespa velutina* and the beewolf *Philanthus triangulum*, for example, possess the basiconic sensilla (Couto et al. 2017; Herzner et al. 2003).

The structural differences between males and females likely reflects their unique biological requirements and behavioral repertoires. The importance of detecting sex-pheromones, for example, is extremely high in males. Accordingly, the honeybee drone antennae are enriched with placoid sensilla that detect the sex-pheromone 9-oxo-trans-2-decenoic acid, and their electrophysiological and behavioral responses to these compounds differ from that of workers (Esslen and Kaissling 1976; Brockmann and Bruckner 2001; Kaissling and Renner 1968; Brockmann, Bruckner, and Crewe 1998; Vetter and Visscher 1997). While more research needs to be done on the precise signaling mechanisms of the peripheral olfactory system, these differences altogether suggest a high level of complexity in terms of the quantity (and perhaps quality) of information that may be processed and how this information is initially encoded within the olfactory system.

Olfactory Processing in the Central Brain

Activation of specific chemosensory receptors by their corresponding ligand leads to depolarization of the ORNs, relaying action potentials to their cognate stereotypic glomeruli in the antennal lobe (AL) (Galizia and Sachse 2010) or, in the case of certain GRs, the subesophageal ganglion (SOG) (Stocker and Schorderet 1981; Nayak and Singh 1983; Shanbhag and Singh 1992; Scott et al. 2001), where projection neurons then carry information to the mushroom body (MB) and the lateral horn (LH). The MB is a key site for integration of information from multiple inputs such as olfaction and taste and the primary center for memory formation and retention. The lateral horn processes information from odor cues related to both learned behaviors and innate responses (Schultzhaus et al. 2017). The cellular logic of olfactory system connectivity is conserved to humans (Hildebrand and Shepherd 1997): despite differences in shape there is deep homology of central brain structures (Strausfeld and Hirth 2013).

As with the peripheral sensory appendages, there are sex- and caste-specific differences in the brain. There are similarly high numbers of glomeruli in the antennal lobe corresponding to the large expansion of chemoreceptors (Nakanishi et al. 2010; Nishikawa et al. 2008; Arnold, Masson, and Budharugsa 1985; McKenzie et al. 2016). The composition of these glomeruli, however, are sexually dimorphic. Both virgin queens and female workers tend to have a comparably high number of glomeruli (Nishikawa et al. 2008; Groh and Rossler 2008). While the number of glomeruli in the male is often substantially lower than that of the females, males often possess exceptionally large structures in the antennal lobe known as macroglomeruli (Nakanishi et al. 2010; Nishikawa et al. 2008; Arnold, Masson, and Budharugsa 1985; Sandoz 2006; Groh and Rossler 2008; Nishino et al. 2009). In other insects, these macroglomeruli clusters are often associated with sex-pheromone detection and processing (Christensen et al. 1995; Kaissling, Hildebrand, and Tumlinson 1989; Hansson et al. 1992; Berg, Zhao, and Wang 2014). This feature is presumably conserved in Hymenoptera, as well (Sandoz 2006; Brockmann and Bruckner 2001; Hansson and Anton 2000; Galizia and Rossler 2010). Curiously, the large female workers in the leaf-cutting ant also possess a macroglomeruli structure which has been hypothesized to be involved in the detection of trail pheromone (Kleineidam et al. 2005).

Another conspicuous feature of the antennal lobe is the absence of particular sensory tracts formed by axons of antennal neurons in the male. The female-specific sensilla basiconica project to the T6 cluster in ants (McKenzie et al. 2016) and the T3 cluster in the honeybee (Kropf et al. 2014). These sensilla detect important pheromones such as CHCs. Males, however, either lack these glomeruli tracts altogether or they are otherwise significantly reduced (Nishino et al. 2009; Nakanishi et al. 2010). While the macroglomeruli in males is likely responsible for the detection of sex-pheromone, the female-specific glomeruli cluster is likely responsible for the processing of odor cues related to colony tasks such as foraging (Kleineidam et al. 2005).

Changes in the brain may also occur as an individual ages and are also observed between different worker castes. The volume of the glomeruli of honeybee workers, for example, changes over the lifetime (Brown, Napper, and Mercer 2004; Sigg, Thompson, and Mercer 1997; Arnold, Budharugsa, and Masson 1988). While these changes in the neuropil may be the result of changes in hormone levels, such as juvenile hormone, as the honeybee transitions from nursing to foraging, activity-dependent experience has also been proposed to profoundly influences glomerular volume (Sigg, Thompson, and Mercer 1997). In *Camponotus*, smaller workers actually appear to have a

higher number of glomeruli than larger workers despite having an overall smaller glomerular volume (Mysore et al. 2009). This may correspond to the increased levels of foraging observed in the minor relative to majors (Simola et al. 2016). However, in the leaf-cutting ant *A. vollenweideri*, the larger workers actually possess a great number of glomeruli and are correspondingly responsible for the majority of foraging within a colony (Kelber C 2010). Altogether, these differences likely reflect the different behavioral repertoires exhibited over ant lifecycles as nurses transition to foragers and between castes.

In addition to neuronal circuits, neurochemical modulators likely to act in the brain to control social behaviors. For example, serotonin and other neuropeptides have modulatory effects on aggression in *Drosophila* (Dierick 2007), whereas dopamine, octopamine and other biogenic amine neurotransmitters are essential to insects in general (Monastirioti 1999; Harold 2007) and are crucial to behavior, learning, and pathological processes in humans and other mammals (Stoesz, Hare, and Snow 2013; Walker and McGlone 2013). Indeed, biogenic amines play a critical role in the collective organization of eusocial insects (Ellen and Mercer 2012; Kamhi and Traniello 2013; Wada-Katsumata, Yamaoka, and Aonuma 2011), whereas octopaminergic neurons directly interact with peripheral chemosensory neurons to regulate male-male aggression (Andrews et al. 2014). The role of biogenic amines in regulating aggression is also conserved in non-insect arthropods (Huber et al. 1997). Both octopaminergic and serotonergic systems regulate social behaviors in ants, including foraging (Muscedere et al. 2011) and NM recognition (Vander Meer, Preston, and Hefetz 2008), and corazonin influences reproductive caste identity in a primitively eusocial ponerine ant *H. saltator* (Gospocic et al. 2017).

Genome Sequencing

While early genome sequencing efforts were dedicated to human and academic model systems such as *D. melanogaster*, considerable progress has been made over the past 15 years to sequence genomes across all of Arthropoda, including Hymenoptera. The honeybee was the first sequenced Hymenopteran genome in 2006 (Consortium 2006), and since that time, more than 50 Hymenopteran genomes have been sequenced with many more currently in progress (Branstetter et al. 2018; Favreau et al. 2018). These efforts have placed Hymenoptera into the heart of a genomic and molecular revolution, where they provide a uniquely nuanced bridge between organismal and ecological studies and pheromone biochemistry.

One of the most prominent features of Hymenoptera genomes are the changes observed throughout the various chemosensory gene families both within Hymenoptera as well as between Hymenoptera and other insect orders. Relative to solitary insect orders such as Diptera, the genomes of ants, bees, and wasps are broadly characterized by large expansions of chemoreceptors (Zhou et al. 2015; Zhou et al. 2012; Smith, Smith, et al. 2011; Robertson and Wanner 2006; Wurm et al. 2011; Oxley et al. 2014; Werren et al. 2010; Smith, Zimin, et al. 2011; Bonasio et al. 2012). At the same time, there are notable subfamilies of insect chemoreceptors that are entirely absent in Hymenoptera genomes. In particular, no orthologs of the dipteran carbon dioxide receptors have been found in the genomes of ants, bees, and wasps. Furthermore, there are diverse and lineage-specific patterns of gene gain and gene loss across Hymenopteran species that are primarily the result of tandem duplication events that yield distinct clusters of chemosensory genes (Zhou et al. 2015; Zhou et al. 2012). Taken together, these findings leave us with more questions than answers, providing the foundation by which to further explore the olfactory genomics of eusociality within the Hymenoptera.

Apocrita

Odorant Receptors

Apocrita genomes contain a significant expansion of OR genes relative to nonsocial insects. Indeed, ants have the largest number of OR genes of any insect species described to date. Perhaps the most notable facet of the highly expanded OR repertoire is a group of receptors collectively referred to as the 9-exon subfamily as these receptors share a similar intron-exon structure. This clade is particularly expanded in ants, representing at least 25% of all their OR genes, and contains a large number of genes whose expression enriched in workers which have been suggested as candidate receptors for CHCs and other chemical cues important for social behaviors (Robertson and Wanner 2006; Bonasio et al. 2010; Wurm et al. 2011; Oxley et al. 2014; Werren et al. 2010; Smith, Zimin, et al. 2011; Zhou et al. 2012; Zhou et al. 2015; Smith, Smith, et al. 2011). However, other OR subfamilies also show differential expansions between insect lineages. For example, the J-subfamily is expanded in honeybees while the U- and F-subfamilies are expanded in ants and wasps, respectively (Zhou et al. 2015). Importantly, the L-subfamily, which represents a substantial number of ORs in the honeybee, also contains the honeybee queen

pheromone receptor AmelOR11 (Wanner et al. 2007), providing additional support that the expansion of ORs has played a critical role in Hymenopteran social evolution.

As for eusocial apocrita, the significant expansion of the OR family may reflect the rich behavioral repertoire unique to highly social species (Robertson and Wanner 2006; Bonasio et al. 2010; Wurm et al. 2011; Oxley et al. 2014; Werren et al. 2010; Smith, Zimin, et al. 2011; Zhou et al. 2015; Zhou et al. 2012; Smith, Smith, et al. 2011). That said, there are several notable exceptions to that hypothesis. To begin, the genomes of several solitary wasp species, including *N. vitripennis* and *M. demolitor*, also possess a large number of OR genes (Robertson, Gadau, and Wanner 2010; Zhou et al. 2015). In fact, these solitary species have more OR and GR genes than the eusocial honeybee *A. mellifera*. On the other hand, all social species sequenced to date have a large number of chemoreceptor genes, and there is evidence of positive selection on these gene families during the transition to eusocial life. Furthermore, genome sequencing of the termite *Z. nevadensis* also revealed a significant expansion of the IR chemoreceptor family mirroring that of the OR family in Hymenoptera (Terrapon et al. 2014).

Altogether, these observations have led to the hypothesis that the rapid birth-and-death evolution exhibited by chemoreceptor families may have led to functional divergences among chemosensory genes such that novel ligand specificity or sensitivity facilitated the adaptation to novel environments (Zhou et al. 2012; Zhou et al. 2015). As the number of chemosensory genes expanded, it provided these insects with the ability to communicate a broader range of information, including social cues. While these changes alone are insufficient for the transition towards eusociality, they have likely played a critical role the evolution of advanced social behaviors.

Gustatory Receptors

While GRs have received relatively less scientific attention compared to the OR family, this important family of chemoreceptors also displays unique and varied patterns of gene birth and death across insect orders and within the apocritan. Curiously, although these insects have retained the ability to detect carbon dioxide (Kremer et al. 2018; Romer, Bollazzi, and Roces 2017; Kleineidam and Tautz 1996), the dipteran carbon dioxide receptors have been lost in apocrita (Robertson and Kent 2009; Zhou et al. 2012). Within Hymenoptera, the number of GR genes present in each species is highly variable. The solitary wasp *C. solmsi* has as few as 5 GRs whereas the invasive fire ant *S. invicta* has 219 (among the highest of any insect) (Zhou et al. 2015). Even

within a single Hymenopteran lineage, there is large variability in GR number. In contrast to the fire ant, the ant *C. obscurior* has a modest number of GRs (34) with other ant species falling somewhere in between. Both honeybees and halictid bees appear to have a reduced number of GRs (10 in *A. mellifera* and 23 in *L. albipes*, respectively).

Ionotropic Receptors

The IR family represents an independent and ancient lineage of chemoreceptor genes in insects that are primarily responsible for the detection of acids and aldehydes (Yao, Ignell, and Carlson 2005; Croset et al. 2010; Benton et al. 2009). Concordantly, IRs genes are more similar within Hymenopteran lineages with relatively stable copy number variation (Zhou et al. 2012). For example, across four different ant genomes (*H. saltator, C. floridanus, L. humile*, and *P. barbatus*), there was only one lineage-specific expansion of IR genes. Namely, the *IR317* subfamily in *C. floridanus*, which expanded from 1 to 7 genes. These results suggest that the IR family may have a more conserved biological role across insect orders although some IRs may also play a role in the detection of species-specific odor cues (Shan et al. 2019).

OBPs and CSPs

The precise role of OBPs and CSPs in peripheral olfactory signaling remains unclear. Historically, these two classes of molecules have been suggested to facilitate the transport and binding of odorant ligands to their cognate receptor (Vogt, Riddiford, and Prestwich 1985). However, knocking out all *Obp* expression in the basiconic sensilla of *Drosophila* does not eliminate responsiveness to either intermittent or prolonged exposure to odorants suggesting that, at the very least, *Obps* may not be necessary for the detection of certain odorants nor the transport of odor molecules (Larter, Sun, and Carlson 2016; Xiao, Sun, and Carlson 2019). Nevertheless, mutant flies showed altered valence thresholds to a linoleic acid sample in a dose-dependent manner in the context of an oviposition dual-choice bioassay suggesting that *Obps* may still be involved in olfactory signaling and corresponding behaviors (Xiao, Sun, and Carlson 2019). Additionally, *Drosophila* lush mutants, which lack *obp76a*, are significantly more attracted to high concentrations of ethanol, propanol, and butanol relative to wildtype flies (Kim, Repp, and Smith 1998; Kim and Smith 2001). At the same time, these mutants are significantly less attracted to the

male-specific phereomone 11-*cis* vaccenyl acetate, and both background firing and responsiveness to this compound are significantly diminished (Xu et al. 2005).

Across Arthropoda, this gene family exhibits relatively high levels of gene birth and death (Vieira and Rozas 2011). Within apocrita, however, both CSPs and OBPs form highly conserved clades with single-copy orthologs found throughout most species and more evolutionarily labile species-specific clades with notably higher levels of gene birth and death (McKenzie, Oxley, and Kronauer 2014). Tissue-specific gene expression analysis revealed that the antennae primarily express only a subset of these olfactory-related genes with a bias towards the conserved orthologs as opposed to the more derived CSPs and OBPs. The other genes may be found in tissues such as the leg and may play a role in the perception of gustatory cues. Alternatively, they may play a more broad role in cuticle biology (Galindo and Smith 2001; Park et al. 2000; Foret, Wanner, and Maleszka 2007).

Despite large expansions in the chemoreceptor families, there appear to be fewer CSPs and OBPs in Hymenoptera relative to other insect orders with more simple olfactory systems. There are approximately 20-30 OBPs and CSPs combined in each apocritan species. *D. melanogaster*, on the other hand, have at least 51 (Hekmat-Scafe et al. 2002). Although importantly, not all of these genes are expected to have a chemosensory function. Taken together, the reduced number of OBP and CSP genes relative to the number of relevant chemical cues used by Hymenoptera and the observation that the conserved orthologs of these families are more likely to show antenna-specific expression patterns suggests that these genes serve a more conserved biological function.

Symphyta

Sawflies are an understudied and oft-overlooked lineage of Hymenopteran insects belonging to the suborder Symphyta. Perhaps part of the reason these insects have received relatively less attention is that, despite the observation that there have been at least seven independent origins of eusociality across ants, bees, and wasps, there are currently no known eusocial sawflies (Wilson and Holldobler 2005). Nevertheless, genome sequencing from one member of this basal group of insects has revealed a number of important insights into the olfactory genomics of Hymenoptera. Consistent with expectations, the genome of the wheat stem sawfly *Cephus cinctus* does not possess a large expansion of ORs relative to Apocrita (Robertson, Waterhouse, et al. 2018). In fact, the *C. cinctus* genome has been reported to contain only 71 genes

encoding tuning ORs alongside the highly conserved coreceptor *Orco*. Of these, a few lineages appear to be unique to Symphyta and absent in Apocrita. Similarly, several OR gene lineages present in Apocrita have been lost in Symphyta. Perhaps the most interesting observation is that the sawfly contains two orthologs of carbon dioxide receptors (*CcinGr24-25*) as well as other GR lineages which have been lost in the apocritan (Robertson, Waterhouse, et al. 2018). There are also a modest number of OR homologs present at the base of various OR subclades in sawflies which have undergone expansions in ants, bees, and wasps. This notably includes *CcinOr69* situated at the base of the 9-exon subfamily and *CcinOr1-9* at the base of a subfamily of genes in *A. meliferra* which contains *AmelOr11*, the putative receptor for the honeybee queen pheromone (Wanner et al. 2007).

Transcriptomics

Antennal sequencing from both males and workers of the species *C. floridanus* and *H. saltator* revealed that, while workers expressed almost nearly all tuning ORs within their repertoire, males only expressed about a third (Zhou et al. 2012). While there were a few male-enriched chemosensory genes, the great majority were upregulated in the workers compared to the male (40 in *C. floriodanus* and 120 in *H. saltator*). Importantly, many of these differentially expressed genes were ORs belonging to the 9-exon subfamily, providing support for the hypothesis that this particular clade may have played a role in the evolution of worker behavior.

As for the two morphological worker castes of *C. floridanus*, there were more similarities than differences. Only 13 differentially expressed ORs were found between minors and majors. All of these genes were upregulated in the minor workers, which may reflect their more prominent role in foraging within the colony. In contrast to OR expression, only a small proportion (15-50%) of GR and IR genes were expressed in either workers or males. There were marginal, if any, differences in expression between males and workers as well as between the minor and major workers of *C. floridanus*. These results highlight the importance of the 9-exon subfamily in worker behavior. Furthermore, the lack of ORs found in the males as well as the diminished expression of the ORs that were present reflect the more specialized role of males in reproduction. In contrast, workers assume a variety of responsibilities within a colony, and are correspondingly better equipped to communicate these chemical cues.

The examination of CSP expression in a related species, *C. japonicus*, identified various genes with high expression levels in the antennae (Hojo et al. 2015). Furthermore, these genes also exhibited differential expression patterns between males, alate (winged) queens, and workers, which suggests they may play a role in either the reproductive biology of the males and queens or the task-associated behaviors of the workers. Curiously, *CjapCSP1*, which has been previously shown to have a binding affinity for CHCs, colocalizes with two ant-associated CSPs—*CjapCSP12* and *CjapCSP13*—in the hydrocarbon responsive basiconic sensilla. Although as discussed above, the precise role of these proteins in olfactory signaling remains unclear (Larter, Sun, and Carlson 2016; Xiao, Sun, and Carlson 2019).

Additional studies on OBP and CSP expression in three other ant species (*C. biroi*, *H. saltator*, and *C. floridanus*) demonstrated a striking similarity in the transcript abundance of various orthologous genes across workers and males of each species (McKenzie, Oxley, and Kronauer 2014). This supports the notion that these OBP and CSP genes likely play a more conserved role in Hymenopteran biology. However, several CSPs belonging to an ant expanded clade of proteins and the paralog group of OBPs show species-specific expression patterns, and perhaps these genes have an even more narrow role in olfaction.

Studies from the honeybee, *Apis mellifera*, reveal similar trends as to those found in ants. When examining differential expression between newly eclosed workers, nurses, and foragers, tuning ORs and GRs were relatively lowly expressed with no significant differences between task groups (Nie et al. 2018). However, OBPs and CSPs were present in high levels, and Obp17 was enriched in nurses relative to age-matched foragers. Furthermore, the homolog CSP1 had the highest expression levels in the antennae among the CSP genes. There were also several P450 genes, carboxylesterases, and glutathione S-transferases that were differentially expressed between these three worker groups and which may function as odorant degredation enzymes (ODEs). Altogether, this data suggests that changes in the chemosensory proteins that regulate activies such as odorant degradation in the sensillar lymph may be corelated to changes in behavior associated with either sex or behavioral task group.

Other transcriptomic studies have examined transcript abundance as an indirect proxy for gene expression in full body extractions. However, many of these full-body studies are prone to false negatives in instances where there may indeed be patterns of tissue-specific gene expression differences or in which different tissue types (e.g. antennae) may have opposing patterns of gene

expression (Johnson, Atallah, and Plachetzki 2013). This is especially problematic with regard to certain olfactory related genes such as chemosensory genes. For example, in light of their dispersed patterns of neuron-specific expression, individual tuning OR genes may have relatively low levels of transcript abundance but nevertheless encode for essential proteins that are extraordinarily capable of supporting olfactory signaling. Therefore, even subtle differences in gene expression may have profound alterations in olfactory signaling. Neverthethess, despite these caveats, the antennal transcriptomic studies described above are likely the more appropriate to look into when considering the regulation of chemosensory gene expression across Hymenopteran castes.

Functional Analyses of Hymenopteran Chemosensory Systems

As the genomes of numerous Hymenopteran species have been sequenced and annotated, the resultant metadata has provided the foundation for applying targeted molecular approaches to this order of insects and shed light on the evolution of olfaction in Hymenoptera. There is now an ongoing effort to link the longstanding interest in Hymenopteran chemical ecology with molecular biology by functionally characterizing the olfactory system, including a better understanding of the odor coding mechanisms involved in pheromone detection as well as identifying the salient chemoreceptors for important chemical cues.

Electrophysiological Studies

Many electrophysiological studies to date have focused on the mechanisms involved in detecting and processing CHC profiles and other pheromones as this represents an important task necessary for reproductive harmony and colony cohesion. In the honeybee, for example, the queen mandibular gland produces a blend of compounds referred to as the queen mandibular pheromone (QMP). QMP influences a variety of behavioral and physiological processes in workers (Higo et al. 1992; Pankiw et al. 1998; Pettis, Winston, and Slessor 1995; Kaminski et al. 1990; Slessor et al. 1988; Pankiw, Winston, and Slessor 1994, 1995). In addition to serving as a sex pheromone to attract males (Brockmann et al. 2006; Gary 1962), the presence of QMP also inhibits ovary development in workers to prevent illegitimate reproductives (Melathopoulos et al. 1996; Pettis et al. 1997; Pettis, Winston, and Collins 1995; Hoover et al. 2003). Using microarray and qPCR, candidate sex pheromone receptors were identified based on their high expression in drone antennae relative to females (Wanner et al. 2007). AmOr11 was subsequently found to detect a

component of the QMP, 9-oxo-2-decenoic acid (9-ODA). Similar studies have also been done in ants. The cuticle of queens and reproductive workers of the ponerine ant *P. inversa* is characterized by relatively high levels of the hydrocarbon 3,11-dimethylheptacosane, and worker antennae display robust responses to this compound (D'Ettorre et al. 2004). In *H. saltator*, *HsOr263*, *HsOr271*, and *HsOr259-L2* have been shown to detect a putative queen pheromone 13,23-dimethylheptatriacontane which can be found on both reproductive queen and gamergate cuticles but is absent from infertile workers (Liebig et al. 2000; Pask et al. 2017; Slone et al. 2017).

Electrophysiological studies of *C. japonicus* first suggested that ants possess a multiporous sensilla dedicated to the detection of non-nestmate cues (Ozaki et al. 2005). The authors observed sensillar responses to non-nestmate CHC extracts. However, this particular sensilla did not respond to nestmate CHC extracts which were very similar in chemical composition with only subtle differences in ratios. These observations lead to the proposal that ants were densensitized and ultimately anosmic to their own colony odor cues. However, more recent studies using both antennal single sensillum electrophysiology and antennal lobe calcium imaging have demonstrated that ants are capable of detecting both nestmate and non-nestmate odor cues (Brandstaetter AS 2011; Brandstaetter 2011; Sharma et al. 2015). Discrete ORN-containing basiconic sensilla are differentially sensitive to alkanes and methyl-branched alkenes, including both queen and worker-enriched hydrocarbons (Sharma et al. 2015). These sensilla also detect a variety of general odorants. This broad-spectrum sensitivity is highly robust, allowing differential detection of enantiomeric CHCs, presumably contributing to the ability of these ants to detect highly complex CHC profiles from both nestmates and non-nestmates.

Pheromone responses also vary between reproductive castes and between males and reproductive females. In the ant *H. saltator*, single sensillum recordings (SSRs) have revealed significant differences in the olfactory responses between workers, gamergates, and males to several straight chain hydrocarbons which comprise caste-specific hydrocarbon profiles (Ghaninia et al. 2017; Ghaninia et al. 2018). These observations likely reflect the distinct behavioral and reproductive requirements among various colony members. For example, *H. saltator* workers, gamergates, and males display distinct CHC profiles (Liebig et al. 2000; Ghaninia et al. 2018). As a worker transitions to a gamergate, a dramatic shift toward longer-chain hydrocarbons occurs, signalling a change in fertility and altering the behaviour of other workers (Liebig et al. 2000). This transition is also accompanied by a dramatic shift in hydrocarbon responses such that

gamergates tend to exhibit decreased responsiveness to hydrocarbons (Ghaninia et al. 2017). This is presumably because gamergates dedicate their time to reproduction while workers carry out tasks that require hydrocarbon signalling such as policing (Endler et al. 2004).

Males, which lack basiconic sensilla, nevertheless possess hydrocarbon-responsive trichoid sensilla (Ghaninia et al. 2018). However, these responses are notably lower and more narrowly tuned than that of workers. In addition to hydrocarbons, the trichoid sensilla also respond to alarm pheromone components whereas the coeloconic sensilla responds to acids, aldehydes, and alcohols consistent with the role of IRs in the coeloconic sensilla of *Drosophila* (Benton 2009, Yao 2005). In contrast, the coeloconic sensilla of females was responsive to a much broader range of odorants, including both general odorants and hydrocarbons. This is consistent with RNA sequencing data demonstrating that males express fewer chemoreceptors relative to workers and typically have much lower chemoreceptor transcript abundance relative to workers with a few exceptions of male-enriched genes (Zhou et al. 2012). That males respond to a small subset of hydrocarbons and general odorants likely reflects their more confined role in reproduction. Males are highly sensitive to a narrow range of chemical cues that are likely involved in aggregating around queens and finding a mate.

Odor Coding and Deorphanization

Various efforts have been undertaken to deorphanize Hymenopteran ORs and identify their biologically salient chemical ligands. Perhaps the two most significant findings to date are the identification of the honeybee AmelOR11 (subfamily L), which detects the queen substance 9-oxo-2-decenoic acid (Wanner et al. 2007), and the jumping ant HsOr263, HsOr271, and HsOr259-L2 (9-exon subfamily), which similarly detect a putative queen pheromone component 13,23-dimethylheptatriacontane (Pask et al. 2017; Slone et al. 2017). In addition, over 40 additional *H. saltator* receptors were heterologously expressed and subsequently tested for their response to a broad panel of straight-chain alkanes and other general odorants (Slone et al. 2017; Pask et al. 2017). Importantly, the detection of CHCs was not restricted to the 9-exon subfamily, but rather broad activation of receptors was seen across the various OR subclades (Slone et al. 2017). Additional studies in ants have also revealed the receptors for 4-methoxyphenylacetone in the jumping ant (HsOR55) and 2,4,5-trimethylthiazole in the Florida carpenter ant (CfOR263).

In another set of studies, the responses of individual *H. saltator* ORs (HsORs) to volatilized hydrophobic CHCs derived from discrete *H. saltator* castes were examined. Each of the 9-exon HsORs examined displayed distinct response profiles to 3 CHC extracts from different castes. Moreover, individual hydrocarbons elicited narrow response spectrums in which structurally-related hydrocarbons that differed only by a single carbon often resulted in dramatic differences in HsOR activity profiles (Pask et al. 2017). Expanding this analysis of HsOR functionality beyond the 9-exon subfamily revealed responses to several classes of semiochemicals, including CHCs as well as a range of pheromones and more traditional "general" odorants (Slone et al. 2017). Taken together, and when viewed through the prism of caste-specific *OR* enrichment as well as distinctive OR subfamily odorant response profiles, these findings suggest that, whereas individual HsORs appear to be narrowly tuned, there is no absolute segregation of tuning responses within any discrete HsOR subfamily. Instead, it seems likely that the *HsOR* gene family as a whole responds to a broad array of compounds that are likely to mediate distinct *H. saltator* behaviors.

In honeybees, the receptors for linalool (AmelOr151) and various other floral compounds (AmelOr152) have been identified (Claudianos et al. 2014). Given the large number of divergent chemosensory genes found across Hymenopteran genomes, there remains a considerable number of opportunities for the characterization of narrowly-tuned and highly specialized receptors that selectively detect important pheromones and other semiochemicals. These findings will continue to inform our understanding of the link between the evolution of the eusociality and social behaviors in Hymenoptera and the role of olfactory signaling in these processes.

Gene Silencing and Editing

Advanced in genetic technologies are crucial for efficient manipulation of genes in ants and other social insects. The most critical breakthrough in this regard was the successful application of CRISPR-Cas9-based targeting in two separate ant model systems. In both *H. saltator* and *O. biroi*, the obligate ORs co-receptor *orco* was knocked out to generate null mutants (Yan et al. 2017; Trible et al. 2017) which displayed a broad spectrum of phenotypes. In *H. saltator*, *a*ntennal retraction bioassays were used to monitor responses to alarm, attractant, or repellent pheromones. While wildtype (WT) ants responded robustly to these odorants, *HsOrco*$^{-/-}$ homozygous mutants displayed significantly lower responses to all odorants compared to heterozygous ants. In addition, several more complex olfactory-driven social behaviors were also

assessed. For example, while newly eclosed WT workers remain in the nest while older workers (>100 days old) leave the nest to forage, young homozygous *HsOrco* worker ants (age <50 days) spent a much longer time outside the nest than WT and heterozygous young workers, suggesting that the *orco* mutation caused a measurable phenotype in a distinct social behavior (leaving the colony), most likely due to a defect in sensing chemical signals which may guide orientation within the nest (Heyman et al. 2017).

Similar patterns were observed in the clonal raider ant *O. biroi* (Trible et al. 2017). Here, responses to both repellents and attractants were disrupted in mutant workers. While odor cues arising from lines drawn with Sharpie™ permanent markers often repel wild-type ants, mutant *O. biroi* workers freely crossed over these boundaries. Mutant workers also failed to follow trail pheromones elicited by the colony. As with *H. saltator*, *orco-/-* individuals exhibited a wandering phenotype and did not cluster together with nestmates. These data show that *Orco* and, by extension *OR*s, exhibit a conserved role in the perception of general odorants and in social behavior plasticity in ants.

Surprisingly, and in contrast to other insect systems where *orco* orthologs have been knocked out (Chiang et al. 2009; Larsson et al. 2004), the loss of *orco*-dependent OR functionality also dramatically impacts the development of the ant antennal lobes into which ORNs project (Yan et al. 2017; Trible et al. 2017). This dramatic change in antennal lobe development mirrors studies in mice, where the absence of neuronal activity leads to developmental defects in the olfactory bulb (Yu et al. 2004). Such profound deficits in the antennal lobe suggests that caution should be taken when interpreting changes in the behavior of mutant workers. Indeed, it is difficult to distinguish whether resultant behavioral phenotypes are the result of a lack of OR signaling or rather any number of differences encountered during development. While these studies represent a substantial stride in our efforts to bring genetic techniques into non-model systems, they also highlight the need for the continued innovation of targeted molecular engineering.

Future Directions on the Study of Olfaction in Hymenoptera

The sequencing of numerous Hymenopteran genomes provided a foundational set of studies which have since facilitated the application of more sophisticated molecular and gene editing techniques. These studies have provided critical insights into the evolution and functional mechanisms of the olfactory sensory system with important implications for our understanding of

the evolution of eusociality and complex social behaviors. While great strides in our understanding of the olfactory genomics in Hymenoptera have been made, there are many outstanding questions to be resolved and many more avenues for future research.

Perhaps the most important avenue for future research is the ongoing development of targeted molecular techniques. Eusocial Hymenoptera represent a significant challenge in this regard due to their long generation time and the production of a largely sterile worker base that makes the establishment of genetic mutant lines difficult. Indeed, the first and only two CRISPR-Cas9 mediated ant mutants to date were performed using evolutionary basal species that have more relaxed constraints on reproduction relative to many other apocritan species (Yan et al. 2017; Trible et al. 2017). *H. saltator* workers transition to reproductive gamergates in the absense of a queen, and *O. biroi* is a queenless ant in which all workers are capable of clonal reproduction. The application of these and other molecular techniques represent an important step towards understanding the olfactory genomics of eusociality.

Epigenetics and the Regulation of Task Allocation

Eusocial Hymenopteran live in sophisticated societies whereby a single genome may give rise to distinct physiological, morphological, and behaviors phenotypes. Therefore, epigenetic regulation of gene expression likely plays a central role in determining various features of social biology such as regulating reproductive hierarchies, caste determination, and task allocation. Broadly, Eukaryotic DNA forms a complex known as chromatin by wrapping around histone protein octamers consisting of four subunits in duplicate—H2A, H2B, H3, and H4 (Luger et al. 1997). This structure serves two primary functions: 1) it facilitates condensation of DNA into the nucleus (Annunziato 2008), and 2) it plays a critical role in transcriptional regulation (Kouzarides 2007). Dynamic covalent post-translational modifications (hPTMs) of the N- and C-termini histone tails alters the structural association between the histone protein and DNA, thereby regulating access of transcriptional proteins to the DNA (Kouzarides 2007). For example, acetylation (ac) leads to a more open state and is associated with increases in gene expression while methylation (me) leads to a more compact state and is associated with decreases in gene expression. In *Drosophila*, *OR* expression in the neuron is determined through a process whereby histone methylation (H3K9me3) silences the expression of all but one receptor (Clowney et al. 2011; Magklara et al. 2011; Sim et al. 2012). Gene expression may also be regulated through

methylation of DNA directly (Cedar and Bergman 2009). In this way, epigenetic modifications alter gene expression such that diverse phenotypes can arise without changing the genome.

In ants and other eusocial insects, epigenetic modifications have been shown to play a key role in morphological development (Alvarado et al. 2015), establishing reproductive castes (Kucharski et al. 2008; Lyko et al. 2010), and behavior (Simola et al. 2016). In *C. floridanus*, worker size correlates with methylation state and expression levels of *Epidermal growth factor receptor* (*Egfr*), a protein ultimately involved in regulating DNA synthesis and cell proliferation (Alvarado et al. 2015). Inhibiting or enhancing genome-wide methylation through the administration of DNA methyltransferase inhibitors or methyl donors led to significant changes in worker size. It is interesting to note that prolonged ingestion by honeybee workers of the royal jelly has been proposed to induce changes in DNA methylation that contribute to queen development (Spannhoff et al. 2011). One protein found in the royal jelly, royalactin, increases body size and ovary development in a process putatively mediated by *Egfr* (Kamakura 2011). More recently, it has been demonstrated that *C. floridanus* minor workers are primarily responsible for foraging behaviors within the colony while majors rarely forage. However, injection of small molecule inhibitors that interfere with hPTMs in callow major workers leads to robust foraging activity that is stable for at least several weeks (Simola et al. 2016). Subsequent studies have revealed that a histone deacetylase inhibitor (TSA) functions alongside a highly conserved neuronal corepressor (CoREST) involved in regulating neuron production to suppress genes that degrade juvenile hormone and promote foraging in injected major workers (Glastad et al. 2019). Together, these results suggest that the epigenetic regulation of gene expression plays a key role across a variety of important aspects of ant physiology and social behavior.

Neuropeptides and the Establishment of a Reproductive Division of Labor

Additional evidence that highlights the importance of gene regulation in eusocial colonies comes from studies using *H. saltator*. In this system, a single genome yields two distinct phenotypes. Workers transition from relatively short-lived non-reproductives that perform tasks such as foraging to longer-lived reproductive gamergates that devote their energy towards reproduction. As this transition occurs within a single workers, it is likely mediated by changes in gene expression. Consistent with this expectation, analysis of gene expression in the brain of workers and gamergates revealed that the neuropeptide corazonin is downregulated in the

reproductives (Gospocic et al. 2017). Injecting corazonin into head of workers who were in the process of transitioning to a gamergate induced worker-like behavior such as foraging while simultaneously suppressing vitellogenin expression in the brain and inhibiting gamergate-like behaviors such as egg laying. Furthermore, RNAi-mediated knockdown of the corazonin receptor (*CrzR*) significantly reduced foraging behavior in workers. In addition to the successful application of molecular techniques such as neuropeptide injection and RNAi, these studies support the role of epigenetic regulation of gene expression in regulating the eusocial division of labor.

Pharmacological Studies and the Odor Coding of Nestmate Recognition

The large expansion of the OR repertoire coupled with the demonstration that several members of this family in *H. saltator* are responsible for the detection of hydrocarbons support the hypothesis that these receptors played a central role in the evolution of social behavior in eusocial Hymenoptera (Robertson and Wanner 2006; Bonasio et al. 2010; Wurm et al. 2011; Oxley et al. 2014; Werren et al. 2010; Smith, Zimin, et al. 2011; Slone et al. 2017; Pask et al. 2017; Zhou et al. 2012; Zhou et al. 2015; Smith, Smith, et al. 2011). These observations lead to the assumption that OR-signaling was involved in the recognition of and subsequent triggering of aggression towards non-nestmates. However, assumptions are not the same as scientific evidence, and a definitive demonstration linking OR function with aggression-mediated nestmate recognition was lacking. It was also unclear whether other sensory pathways, such as gustation or vision, were also necessary for recognition to occur.

The lack of scientific evidence in this regard was largely the result of two fundamental problems. First, certain ant species such as *H. saltator* do not actively aggress con-specific non-nestmates. Rather, barring conflicts between multiple reproductive gamergates, colonies can be readily split or fused together. This unique aspect of *H. saltator* biology facilitated the application of CRISPR-Cas9 but necessarily exclude certain behavioral studies. Second, the profound alterations in the neuroanatomical development of the antennal lobe of $orco^{-/-}$ mutants presents a significant confounding variable (Yan et al. 2017; Trible et al. 2017). It is not possible to distinguish whether certain behavioral changes are due to the loss of OR-signaling, modifications in the structure and function of the brain, and/or alterations in their developmental experiences. These problems highlight a few of the exceptional difficulties that arise when applying molecular approaches to Hymenoptera and emphasize the need for their continued development.

One promising alternative to genetic engineering is the use of pharmacological compounds to disrupt various biological functions. One such set of compounds are a group of triazol-based derivatives that modulate OR activity (Jones et al. 2011; Jones et al. 2012; Rinker et al. 2012; Taylor et al. 2012; Romaine et al. 2014; Pask et al. 2011). These compounds, which include allosteric antagonists (VUANT) and allosteric agonists (VUAA), selectively interact with ORCO to inhibit or stimulate olfactory signaling, respectively. In light of the confounding variables that are introduced when generating *orco* null mutant ants, these VU-class compounds provide a powerful tool for manipulating orco protein function in otherwise wild-type individuals. Furthermore, these orco modulators have been successfully applied across a wide range of insect orders, including Hymenoptera. For example, in the carpenter ant, *C. floridanus*, exposure to volatilized VU-class compounds significantly reduces aggression between non-nestmates without altering aggression towards nestmates (Ferguson et al. 2020). These results highlight the importance of OR-signaling in mediating nestmate recognition.

Chapter II. Functional characterization odorant receptors in the ponerine ant, *Harpegnathos saltator*[†]

Abstract

Animals use a variety of sensory modalities—including visual, acoustic, and chemical—to sense their environment and interact with both conspecifics and other species. Such communication is especially critical in eusocial insects such as honey bees and ants, where cooperation is critical for survival and reproductive success. Various classes of chemoreceptors have been hypothesized to play essential roles in the origin and evolution of eusociality in ants, through their functional roles in pheromone detection that characterizes reproductive status and colony membership. To better understand the molecular mechanisms by which chemoreceptors regulate social behaviors, we investigated the roles of a critical class of chemoreceptors, the odorant receptors (ORs), from the ponerine ant *Harpegnathos saltator* in detecting cuticular hydrocarbon pheromones. In light of the massive OR expansion in ants (~400 genes per species), a representative survey based on phylogenetic and transcriptomic criteria was carried out across discrete odorant receptor subfamilies. Responses to several classes of semiochemicals are described, including cuticular hydrocarbons and mandibular gland components that act as *H. saltator* pheromones, and a range of more traditional general odorants. When viewed through the prism of caste-specific OR enrichment and distinctive OR subfamily odorant response profiles, our findings suggest that whereas individual *HsOr*s appear to be narrowly tuned, there is no apparent segregation of tuning responses within any discrete *HsOr* subfamily. Instead, the HsOR gene family as a whole responds to a broad array of compounds, including both cuticular hydrocarbons and general odorants that are likely to mediate distinct behaviors.

Significance

The tuning of odorant receptors to their particular odorants is crucial for better understanding of how olfactory cues mediate ant social interactions. To help decode the olfactory system of ants, a selection of odorant receptors (ORs) from several phylogenetically distinct

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subfamilies from the ponerine ant *Harpegnathos saltator* were tested against a panel of ant semiochemicals. Responses were observed to both cuticular hydrocarbon components, some of which are known pheromones, and "general odorants," demonstrating broad coverage of these odor spaces across several subfamilies of receptors. These results do not align with currently held hypotheses of OR subfamily odor coding and provide further insight into the evolution of pheromone perception within ant clades and the role this plays in complex social behaviors.

Introduction

The detection of ecologically relevant chemosensory information is critical to the survival and propagation of all organisms. For example, sex pheromones allow members of the same species to locate and assess mates, and predators use volatile kairomones to locate prey. There is long-standing interest in understanding the pheromonal communication of insects and, in particular, exploring how semiochemicals govern the interactions of eusocial colonies. Ants are intriguing for the purposes of chemosensory studies, because of their diversity and exploitation of cuticular hydrocarbons (CHCs) for nest-mate recognition, and as signals of reproductive and caste status. Most ants live in closed societies within a shared colony or nest—with stereotypic social behaviors that involve a strict division of reproductive labor—in which multiple overlapping generations of sterile workers cooperate to nurture the progeny produced by the reproductives, which usually consist of single or small numbers of long-lived, highly fertile queens and short-lived male drones (Crespi and Yanega 1995). Reproductive status within the colony is thought to be signaled primarily by a subset of the hydrocarbons secreted onto the external cuticle of insects and other arthropods (e.g. ref. (Monnin 2006)) that also function to maintain water balance (Gibbs 1998). In fact, colony identity is conveyed by a highly diverse set of CHCs, and intraspecific and interspecific invaders from other colonies are detected and defended against as a consequence of having a different CHC blend than the blend associated with a particular nest/colony (van Zweden, Dreier, and d'Ettorre 2009). In addition, other non-CHC olfactory stimuli play important roles in ant chemical ecology as alarm, trail, or recognition pheromones and are often found in ant exocrine glands (do Nascimento, Billen, and Morgan 1993) and in the microbiota of the ant cuticle (Dosmann, Bahet, and Gordon 2016).

Although numerous ant species are being used as research models, the ponerine ant *Harpegnathos saltator* possesses several advantages that make it an ideal species for study.

Notably, its basic social and chemosensory behaviors have been described in detail (Peeters and Hölldobler 1995). Perhaps more critically, *Harpegnathos* workers can, under certain circumstances, convert into gamergates (from the Greek for "married worker"). As such, *H. saltator* represents a genetically tractable model system for studying social organization in an insect society.

Despite a rapidly developing body of knowledge on the phylogenetics of ant chemoreceptors (Zhou et al. 2015; Zhou et al. 2012), the molecular elements that are responsible for the detection of ant pheromones remain largely uncharacterized. As is the case for other insects, the *H. saltator* genome contains three major classes of chemoreceptors—odorant receptors (ORs), gustatory receptors (GRs), and variant ionotropic receptors (IRs)—and several other receptor classes such as TRP channels, which also have been shown to have chemosensory roles, reviewed in ref. (Suh, Bohbot, and Zwiebel 2014).

Within the ant clade, the highly expanded OR superfamily displays a striking degree of divergence (Zhou et al. 2015; Zhou et al. 2012), suggesting that the detection of ant pheromones—and of CHCs in particular—is largely mediated by these diverse chemosensory receptors. In fact, the role of ORs in queen pheromone perception has already been confirmed in another eusocial hymenopteran— the honey bee *Apis melifera* (Wanner et al. 2007). Functionally, insect OR complexes consist of an odorant coreceptor subunit (Orco), necessary for the trafficking and function of the complex, and a highly divergent "tuning OR" (ORx) that determines the odorant specificity of the complex (reviewed in ref. (Suh, Bohbot, and Zwiebel 2014)).

In Diptera, each odorant receptor neuron (ORN) is believed to generally express a single tuning *Or* gene, which determines the odorant specificity of the ORN, and each olfactory sensillum generally houses 1–4 ORNs (Stocker 1994). In contrast, ant sensilla are far more complex. In particular, female (worker) specific sensilla basiconica that have been shown to detect CHCs potentially contain in excess of 130 ORNs per sensillum (Nakanishi et al. 2009).

Previous work has strongly implicated the nine-exon subfamily of ORs, which makes up nearly 30% of the 347 putative *Or* genes in *H. saltator* genome (*HsOr*s) and is highly expanded within the ant lineage (Zhou et al. 2015; Zhou et al. 2012), in the detection of CHCs, based on their enrichment along the hydrocarbon-sensitive ventral portion of the worker antennae (McKenzie et al. 2016). Transgenic expression of a subset of these genes in *Drosophila* olfactory sensilla confers receptor-specific responses to a panel of CHCs. However, because other OR

subfamilies are also expanded in ant lineages, there is the possibility that CHCs may also be detected by ORs outside of the nine-exon subfamily. To address this question, we have functionally characterized 25 distinct *HsOr*s spread across 9 OR subfamilies by using heterologous expression in *Drosophila melanogaster* antennal ORNs, which has proven to be amenable as an in vivo heterologous expression system for insect chemoreceptors. These receptors were further classified based on their enrichment in male versus worker antennae (Zhou et al. 2012), because differentially abundant ORs are likely to underlie distinct pheromonal signaling pathways in ants. An understanding of the functional responses of these diverse receptors to multiple classes of compounds—CHC-associated hydrocarbons, mandibular gland components, and importantly, general odorants—provides significant insight into the chemical ecology of *H. saltator* that extends our understanding of the functionality of the expanded family of ant ORs.

Results and Discussion

To begin to understand the molecular components that facilitate the distinctive social interactions exhibited by different ant castes, we examined the responses of ORs to a variety of social and environmental stimuli. This endeavor was facilitated by the identification of the complete OR repertoire from several species of ants, which revealed that ants possess some of the largest tuning OR repertoires identified to date. The characterization of the odorant responses of these peripheral ORs represents the initial step in understanding the molecular processes that underlie the detection of CHCs and other semiochemicals by *H. saltator*. Although this report focuses on ORs, it is likely that additional non-OR chemosensory components may also play important roles in the perception of social pheromones.

We prioritized the characterization of *HsOr*s that showed enrichment in antennae of males and workers or which belong to OR subfamilies showing significant patterns of positive selection or gene birth and death in ants or eusocial hymenopterans (Figure II-1A**Error! Reference source not found.**) (Zhou et al. 2015) because these subfamilies are potentially likely to encompass *HsOr*s with species-specific functionality often associated with pheromones. Within those parameters, preference was given to *HsOr*s that lie phylogenetically outside of the nine-exon subfamily in light of the functional characterization of 22 members of that *HsOr* subfamily in a parallel study (Pask et al. 2017). HsORs were tested against commercially available alkanes and other compounds known to be present on *H. saltator* cuticle or in exocrine glands and constituents of our in-house

chemical screening library that encompass a selection of general odorants across diverse chemical classes that are commonly tested in insect olfactory systems. This base panel of ~70 odorants spans a broad chemical space known to play a role in a diverse set of ant behaviors and would allow rapid identification of OR/ligand relationships with a high likelihood of biological relevance.

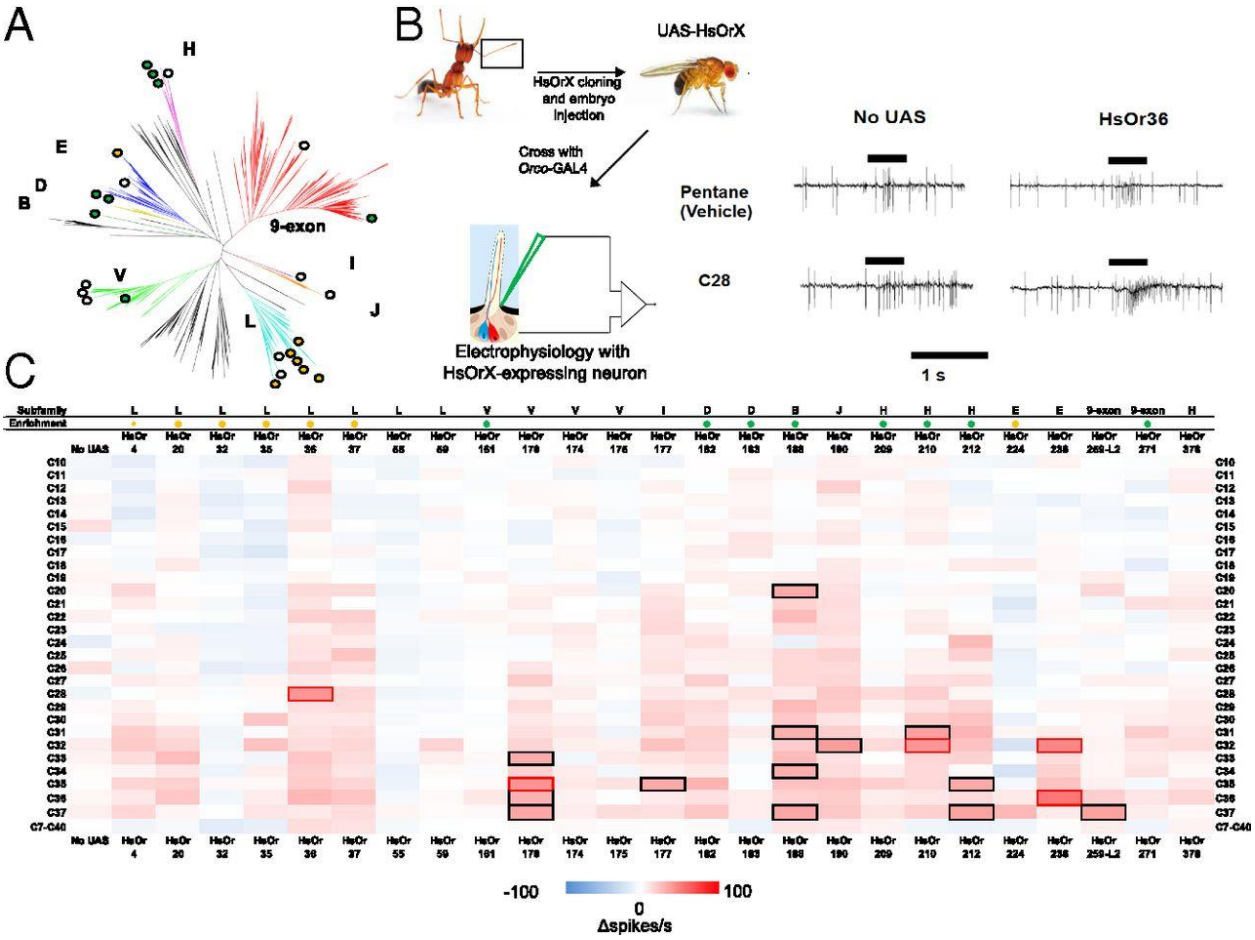

Figure II-1. SSR responses of HsOR receptors to 28 cuticular hydrocarbons and a hydrocarbon mixture.
SSR responses of HsOR receptors to 28 cuticular hydrocarbons and a hydrocarbon mixture. (*A*) Summary of the phylogenetic relationship of the *HsOr* subfamilies and the receptors examined in this study. (*B*) Schematic summarizing SSR technique, along with sample traces comparing responses to heated control (pentane) with responses to heated cuticular hydrocarbons. (*C*) Heat map of responses in ab2A neurons to each hydrocarbon. Responses are calculated as the change in spike frequency induced by each stimulus, relative to the prestimulus spike frequency (after subtraction of the pentane control). The subfamily identity for each *HsOr* is indicated at the top, along with enhanced transcript abundance (Zhou et al. 2012) for workers (orange dots) and males (green dots). Responses above 30 spikes per s are indicated by black boxes, and responses above 40 spikes per s are indicated by red boxes.

Responses to Cuticular Hydrocarbons in Single-Sensillum Drosophila Recordings.

We conducted an initial screen for hydrocarbon responses among our candidate *HsOr*s by assembling a stimulus panel of straight chain alkanes spanning C10 to C37 and testing them against transgenic flies expressing *HsOr*s of interest in ORNs where they can form functional heteromeric complexes with endogenous Orco coreceptors. Hydrocarbon stimuli were volatilized before application by using a brief heat pulse (Materials and Methods). We used single-sensillum recordings (SSRs) from individual antennal sensilla and found that the *Drosophila* ab2 sensillum displayed minimal background response to volatilized hydrocarbons or solvent (Figure II-1B), rendering this sensillum ideal for our investigations of HsOR-mediated hydrocarbon responses.

To sort potential CHC responses from nonresponses, we initially set a threshold of at least 30 spikes per s, which is six times higher than the spontaneous firing rate of ab2A (de Bruyne, Foster, and Carlson 2001). Using this response threshold, we identified 18 HsOR-ligand combinations across 9 HsORs that were responsive to alkane ligands (Figure II-1). Further normalizing these responses by subtracting the "no-UAS" (i.e., from parental flies with only *Orco-GAL4* containing chromosomes) control response for each hydrocarbon produced only minor changes in the overall results, with 17 of the 18 suprathreshold HsOR/hydrocarbon combinations exceeding the >30 spikes per s threshold (Figure A-1). Most strikingly, 17 of these 18 HsOR-hydrocarbon combinations were for hydrocarbons with a chain length of C28 or longer, suggesting a tuning bias toward longer-chain alkanes, consistent with our observation of odor coding within the nine-exon *HsOr* subfamily (Pask et al. 2017). The single exception was *HsOr*188, which showed a suprathreshold response to C20. It is noteworthy that this gene is the only known member of the ant OR subfamily B in *H. saltator*, which has relatively few members (1 to 2 genes) in all ant genomes examined thus far (Zhou et al. 2015). The tight restriction of subfamily B members is maintained across species, suggesting they may have a highly conserved and relatively narrow role in ant chemosensory processes, although we can still only speculate as to whether that role is primarily as a detector for the shorter chain hydrocarbon C20. This sensitivity and others detailed in this report are interesting in light of the CHC biosynthetic pathways, which renders even-numbered straight-chain hydrocarbons generally much less abundant on insect cuticles than odd-numbered chains, although it must be stated that even low-abundance signals can function as powerful pheromones depending on the sensitivity of the corresponding receptor.

*HsOr*36, 210, 170, and 236 responded robustly to hydrocarbon stimuli with chain lengths of C28 or longer. *HsOr*36, a subfamily L receptor whose transcript shows an ~6.4-fold enrichment in male antennae over worker antennae (Zhou et al. 2012), was the most intriguing. HsOr36 responded strongly and specifically to C28 at >40 spikes per s, with no other responses that reached our 30 spikes per s threshold. In contrast, another subfamily H receptor, *HsOr*210, showed a highly significant, 46-fold enrichment in worker antennae compared with males and a suprathreshold response to C32. The third receptor in question, *HsOr*170, is a subfamily V receptor with low and equal mRNA levels in antennae of workers and males, which elicited a suprathreshold response to C35 along with responses slightly below the 30 spike per s cutoff to C33, C36, and C37. Finally, the fourth receptor, *HsOr*236, is a subfamily E receptor that also showed equal transcript abundance between antennae of workers and males. However, the response profile of *HsOr*236 was remarkable in having two distinct responses above 40 spikes per second to even-numbered alkanes—one to C32, and another to C36 (Figure II-2A). These responses were clearly absent in control lines without the UAS-*HsOr*236 transgene (Figure II-2B). As an additional validation, all receptor-ligand combinations above 40 spikes per s (including *HsOr*236 and C36) were retested within a dose–response paradigm, revealing a clear concentration dependency (Figure II-2C-G).

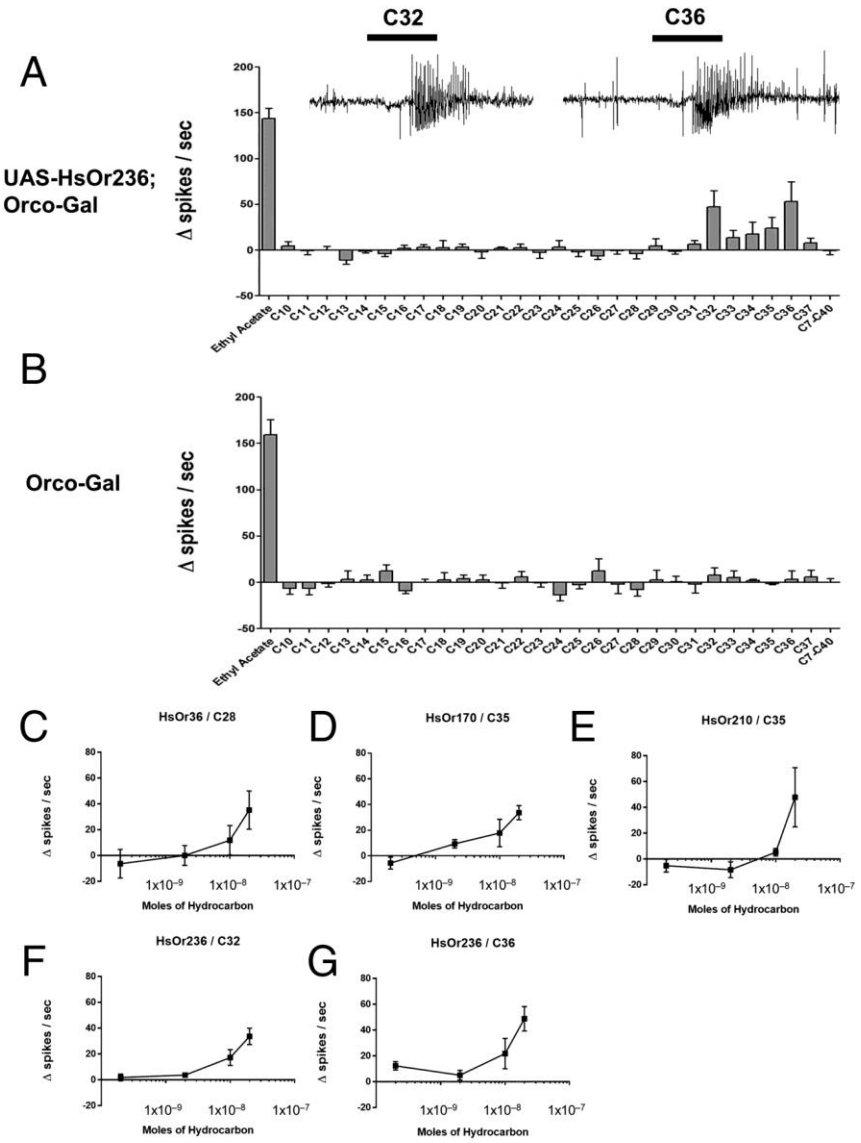

Figure II-2. Characteristic response of an HsOR receptor to cuticular hydrocarbons by SSR. (A) Example of an odorant receptor, HsOr236, which shows responses to specific CHCs by using the heated-puffing protocol. Characteristic response of an HsOR receptor to cuticular hydrocarbons by SSR. (*A*) Example of an odorant receptor, HsOr236, which shows responses to specific CHCs by using the heated-puffing protocol. (*B*) Normal *Drosophila* ORNs show no response to CHCs. Ethyl acetate (the leftmost odorant shown) is a control odorant that activates the native *Drosophila* odorant receptor in the ab2A neuron (n = 5 for *A* and *B*, and error bars are SEM). (*C*–*G*) CHC responses shows dose dependency. The response of all receptor-hydrocarbon combinations >40 spikes per s in Figure II-1 were retested across four different doses: 0.2, 2, 10, and 20 nmol (n = 5, and error bars are SEM). In each case, the magnitude of response showed a clear correlation with the dose of hydrocarbon stimulus.

We next conducted a more quantitative analysis to assess significant differences in HsOR-mediated alkane responses compared with no-UAS controls by using a parametric one-way ANOVA with correction for multiple comparisons and a two-stage step-up method (Benjamini, Krieger, and Yekutieli 2006) at a 0.10 false discovery rate (FDR). Using this criteria, we identified

nine *HsOr*s outside of the nine-exon subfamily that mediate significant excitatory (Zhou et al. 2015) or inhibitory (Crespi and Yanega 1995) responses to hydrocarbon stimuli (Figure A-2). A caveat to analyzing large electrophysiological datasets using strict statistical analysis that corrects for many comparisons is that potentially meaningful discoveries may be overlooked because of modest replication number. It is noteworthy that although no hydrocarbon responses were identified in the nine-exon subfamily through this quantitative analysis, we found that HsOr259-L2 had a sixfold higher response to C37 relative to controls.

Nevertheless, this broader analysis further supports and indeed extends our observation that HsOR-mediated responses to hydrocarbons are not, as previously hypothesized (Zhou et al. 2015; Zhou et al. 2012; McKenzie et al. 2016), restricted to the nine-exon *HsOr* gene subfamily. Furthermore, within the subset of statistically significant responses, we discovered a strong bias toward the longer chain alkanes commonly found in *H. saltator* CHCs (do Nascimento, Billen, and Morgan 1993; Liebig et al. 2000). Indeed, the majority of CHCs that have thus far been identified on cuticles of *H. saltator* workers and reproductives are between 28 and 37 carbons in length, although a CHC with 23 carbons has been reported (Liebig et al. 2000), and antennal responses to hydrocarbons as small as decane (C10) have been observed from antennae of workers (Ghaninia et al. 2017). This observation is consistent with the current paradigm that hydrocarbons play an essential role in signaling colony membership, social status, or other characteristics. If the long chain-sensitive HsORs characterized here function as biological detectors of CHC-based social pheromones, it would make sense that their sensitivity would mirror the narrow range of CHCs which *H. saltator* actually produces. Alternatively, it is possible that the molecular receptors for biologically salient short-chain hydrocarbons are among the *HsOrs* that remain functionally uncharacterized.

Another interesting aspect of our study is the significant sensitivity to hydrocarbons within a distinctive group of male-enriched *HsOr*s. This result would suggest that CHCs are not only used as pheromones to regulate social interactions between workers, gamergates, and queens in *H. saltator*, but may also be used to regulate social interactions between reproductive females and males (i.e., mating pheromones) or perhaps another class of semiochemicals with particular relevance to male biology. This finding is consistent with the recent report that CHCs are extensively used as sex pheromones throughout the Hymenoptera (Kather and Martin 2015).

To expand the range of hydrocarbons in our odorant panel, we obtained 11 different alkenes and custom-synthesized methyl-branched hydrocarbons that are found among *Harpegnathos* CHCs (do Nascimento, Billen, and Morgan 1993; Liebig et al. 2000). These hydrocarbons were initially used to test responses from the two nine-exon HsORs in our receptor collection, which, based on phylogenetic and transcriptomic considerations, is hypothesized to be the *HsOr* gene subfamily most likely to detect CHC pheromones involved in eusociality (Zhou et al. 2015; Zhou et al. 2012; McKenzie et al. 2016). Of these receptors, *HsOr*271 displayed a strong response (60 spikes per s, Figure II-3) to 13,23-dimethyl-C37, which has been implicated as part of the fertility signal in *H. saltator* (i.e., the "queen pheromone") (Liebig et al. 2000). The expression of HsOr271, as is the case for many of the nine-exon receptors, is consistent with a role in the detection of reproductives by workers, as it is enriched ~175-fold in the antennae of workers relative to antennae of males (with fragments per kilobase million values of 24.7404 and 0.14068, respectively) (Zhou et al. 2012). In addition to *HsOr*271, a newly identified paralog of the nine-exon family member *HsOr*259, *HsOr*259-L2, also displayed a weaker response to the 13,23-dimethyl-C37 component of the fertility signal (31.8 spikes per second; Figure II-3), although it should be noted that this particular receptor showed a similarly strong response to C37 (36.6 spikes per second). These results suggest there may be multiple receptors with some level of tuning/sensitivity to this dimethyl queen pheromone, perhaps reflective of combinatorial interactions for gradient navigation and strong and redundant sensitivity to this important semiochemical.

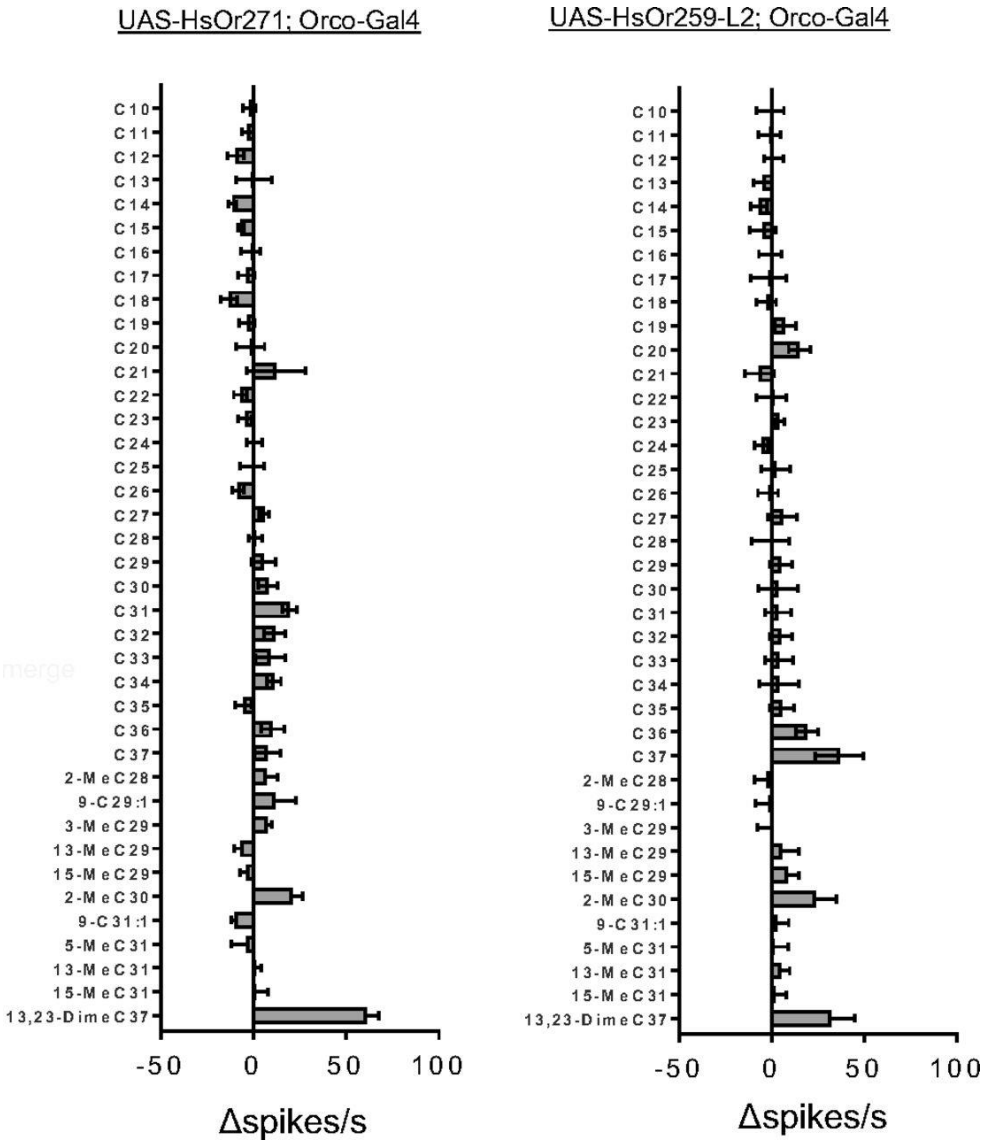

Figure II-3. Responses of two nine-exon HsOR receptors to a panel of branched-chain alkanes and alkenes. Responses of two nine-exon HsOR receptors to a panel of branched-chain alkanes and alkenes. The 11 alkenes and branched-chain alkanes tested are known constituents on *Harpegnathos* worker and/or gamergate cuticle, including the queen pheromone 13,23-dimethylheptatriacontane. $n = 5$, and error bars are SEM.

General Odorant Responses in Drosophila Electroantennogram Recordings.

To expand our analysis beyond hydrocarbons, we next examined nine-exon and nonnine-exon *HsOr*-mediated responses to a stimulus panel comprising an additional 40 non-CHC volatiles across a broad range of general chemical space. To accomplish this survey, we used a whole-field electroantennogram (EAG) recording paradigm that provides high-throughput ability to broadly survey the whole antennae for physiological responses. Although both EAGs and SSRs reveal stimulation and inhibition of antennal ORNs (Figure A-1), it is important to note

that our SSRs were narrowly focused on the ab2A ORN, which endogenously expresses *DmOr*59b. In *Drosophila*, *DmOr*59b is a broadly tuned receptor responding to many general odorants that, in this context, would mask the activity of exogenous *HsOr* transgenes (Ueira-Vieira et al. 2014). EAGs also allowed us to more fully exploit the ability to express *HsOr* transgenes throughout the antennae. Furthermore, the constituents of the general odor panel are much more volatile than CHCs, facilitating their delivery to the antennae as headspace volatiles. This feature removed the constraint of heat-assisted delivery that is required for CHCs and which generates significant whole antennal background activity.

As expected, the raw EAG responses were generally positive for all stimuli tested, likely due to the endogenous activity of the *Drosophila* chemosensory system that can be seen in the no-UAS parental background control antennae. To account for these responses, we used an additional level of normalization by subtracting the responses of the endogenous *Drosophila* receptors in the antenna in the Orco-Gal4 background from the stimulus responses (Figure II-4). After this normalization, the responses to most odorant stimuli were remarkably consistent across transgenic fly lines, with nearly all UAS-*HsOr* transgenes, notably including the two nine-exon HsORs in our test panel (*HsOr*259-L2 and HsOr271), facilitating odorant responses that were greater than (stimulatory) or, in many instances, less than (inhibitory) endogenous responses observed in the Orco-Gal4 background flies (within ±50%). That said, even with this treatment, several potential artifacts must be acknowledged. First, the simplest carboxylic acids—methanoic (formic) and ethanoic (acetic) acid—showed significantly reduced (inhibitory) responses relative to diluent alone control (paraffin oil), which given their intensity, potentially reflect recording artifacts induced in the antennae and/or recording electrodes by the chemical nature of these acids, a phenomenon that has been occasionally reported by other groups (Rollmann, Mackay, and Anholt 2005). This observation can likely be attributed to the high volatility of these acids that potentially could give rise to massive, nonbiologically relevant, odorant concentrations being delivered to the antenna. This effect may be exacerbated by the low solubility of such polar compounds in the paraffin oil diluent. Second, pentanol elicited the highest response observed to any odorant in most of the lines tested, that varied from two to five times the paraffin oil control response (before normalization to the Orco-Gal4 control). We attribute this response to the placement of the glass recording electrode proximate to the distal

end of the *Drosophila* antenna, which contains high numbers of the pentanol-responsive at2 sensillum (Hallem and Carlson 2006).

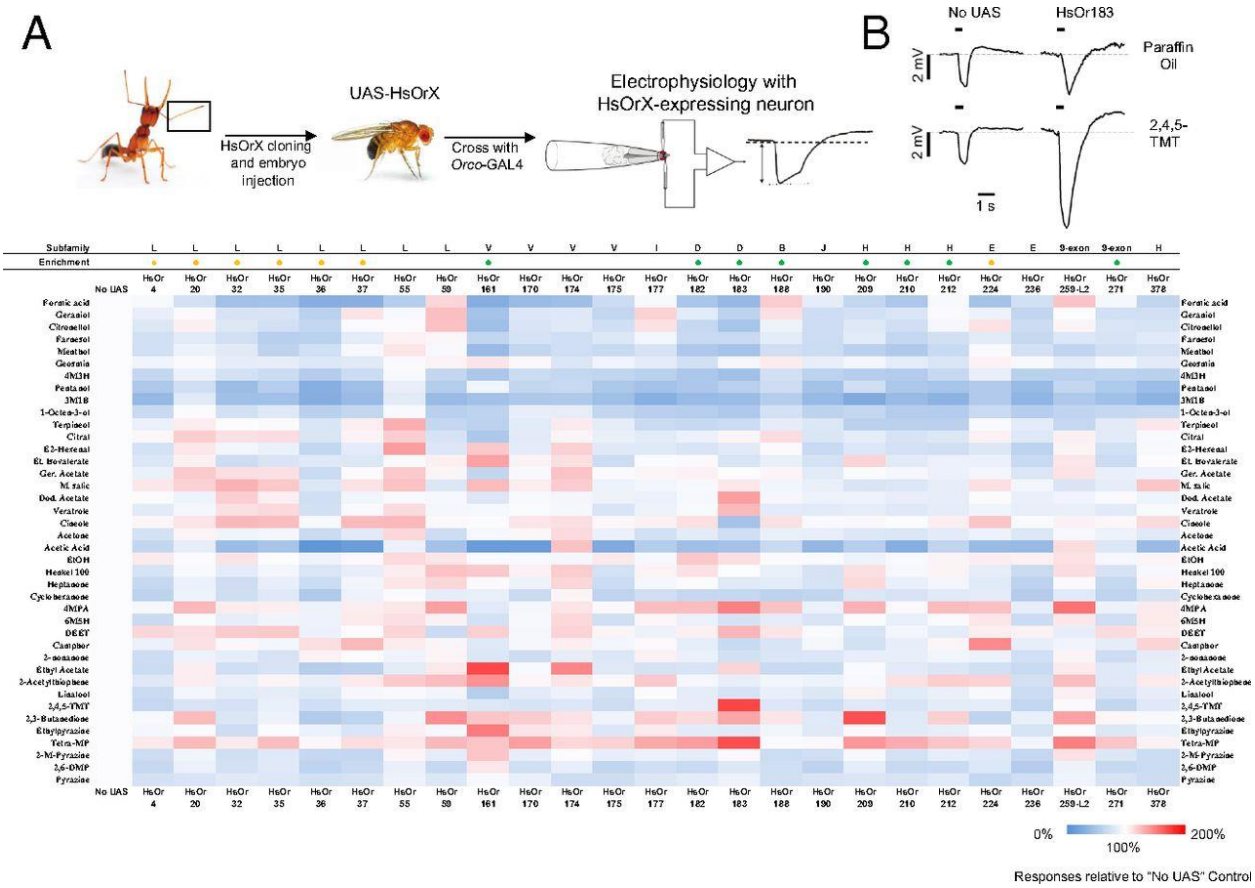

Figure II-4. EAG responses of HsOR receptors to volatile odorants.
EAG responses of HsOR receptors to volatile odorants. (*A*) Schematic summarizing electroantennogram recording technique, along with sample traces comparing responses to diluent alone (paraffin oil) and the volatile odorant 2,4,5-trimethylthiazole. (*B*) Heat map of EAG responses. Data shown is the same as Figure A-3, with an additional round of normalization to the no-UAS control line. Responses are a percentage value relative to the no-UAS line.

In advance of quantitative analyses, several aspects of these odorant responses bear discussion. Most notably, four *HsOr*s displayed stimulatory responses >1.6 times greater than the Orco-Gal4 parental control flies (Figure II-4). *HsOr*59 (a subfamily L receptor), *HsOr*161 (a subfamily V receptor that is 5.6 times enriched in worker versus male antennae) (Zhou et al. 2012), *HsOr*183 (subfamily D), and *HsOr*209 (subfamily H) exhibited the largest responses to this panel of 40 generic volatile odorants compared with Orco-Gal4 endogenous control flies. *HsOr*161 transgenes elicited robust responses to ethyl acetate that were ~1.7 fold higher than those of Orco-

Gal4 parental control flies, and significant inhibitory responses (<50% of the endogenous response) to the *Harpegnathos* mandibular gland pheromone 3-methyl-1-butanol (3M1B) (do Nascimento, Billen, and Morgan 1993) and the plant-based insect repellents menthol, citronellol, geraniol, and citral (Figure II-4). Inhibition by some ligands and activation by others is a common phenomenon among chemoreceptors and could indicate that *HsOr*161 is a relatively broadly tuned receptor. In contrast, transgenic flies expressing antennal *HsOr*59 were significantly stimulated by citronellol and geraniol as well as formic acid (a formicine ant alarm pheromone) and 2,3-butanedione, further demonstrating that our EAG system is not intrinsically biased against detecting stimulatory responses.

Similarly, *HsOr*183 exhibited strong responses to 2,3,5,6-tetramethylpyrazine (Tetra-MP or ligustrazine) and 2,4,5-trimethylthiazole, whereas 2,3-butanedione elicited strong responses from *HsOr*209. We also examined responses to the widely used insect repellent *N*,*N*-diethyl-*meta*-toluamide (DEET), which has been proposed to act as both an activator and an inhibitor of insect ORs. DEET elicited only modest responses from a subset of the *HsOr*s tested here. Of these receptors, *HsOr183* showed the strongest response to the chemical (an increase of ∼28% relative to no-UAS control).

To examine general odorant response data more quantitatively, we tested for significantly different responses to general odorants between the no-UAS control and each *HsOr*. As before, we used a parametric one-way ANOVA with FDR correction. This analysis yielded far more significant inhibitory responses and a surprising number of excitatory responses (Figure A-3). Indeed, the broad range of significant inhibitory responses extended across all of the *HsOr*s tested. Whereas odor-evoked inhibitory responses have biological significance insofar as odor coding, it is also possible that a fraction of the widespread inhibition seen in this analysis is an artifact of the overexpression of *HsOr* transgenes across all antennal ORNs. To better understand the variance in our odorant response profiles, we also carried out a principal component analysis (PCA) of all of the *HsOr*-mediated responses to the different classes of ligands tested (Appendix B, Figure A-4 and Figure A-5, Table A-1, and Table A-2).

Overall, it is notable that the *HsOr*s showing the strongest EAG responses to general odorants are distinct from those that exhibited the strongest SSR responses to hydrocarbons. Thus, whereas *HsOr*s as a whole are broadly tuned to both volatile odorants and hydrocarbons, individual receptors appear to be relatively narrowly tuned to specific ligands. This observation is consistent

with the lack of widespread EAG responses to the "Henkel 100," a standardized mixture of 100 distinct general odorants that might be predicted to robustly activate broadly tuned receptors. In contrast, in our studies this blend elicited only a modest activation (never exceeding ~20% increase over the native no-UAS response) for a single nine-exon family member ($HsOr259_{L2}$) and five nonnine-exon *HsOr*s *(HsOr59, 161, 174, 182, 209*), none of which were quantitatively significant across the entire panel of *HsOr*s tested. Because the *H. saltator* genome contains almost 400 *HsOr* genes, the combinatorial capacity of this repertoire is likely to collectively provide broad coverage across the biologically relevant odor space although individual receptors, when viewed unilaterally, may be narrowly tuned.

Conclusions

This study provides additional support for the further development of *H. saltator* as an insect model for the study of the molecular basis of olfactory signaling, pheromone detection, and more broadly, the underlying mechanistic bases for social behavior. A significant aspect of those questions focuses on the role of peripheral chemosensory receptors, with particular emphasis on the rapidly evolving OR superfamily that is greatly expanded in the genomes of highly social insects. Among those *HsOr*s we interrogated with short and long-chain hydrocarbons, the response of the male-enriched odorant receptor *HsOr*36 is perhaps the most intriguing, given that male ants are generally thought to have little social interaction with the rest of the colony outside of mating. Octacosane (C28), a strong ligand for *HsOr*36, has no specifically defined role in *Harpegnathos*; although it is found on the cuticle of *Harpegnathos* workers and reproductive (Liebig et al. 2000), and on the cuticles of distantly related ants such as *Linepithema humile* (Liang, Blomquist, and Silverman 2001), the absolute abundance is likely quite low because of the biosynthetic constraints on even-numbered carbon chains.

Although it is unknown whether *Harpegnathos* female reproductives actually use octacosane or other CHCs as sex pheromones to attract males, it should be recognized that male ants are often promiscuous in their mating choices. In fact, males from some ant species will even mate with heterospecific queens—a fact that is often exploited by such queens to produce additional sterile workers (Umphrey 2006). The response of the nine-exon receptor *HsOr*271 to the queen pheromone 13,23-dimethyl-C37 is also notable, although it is also possible that there

are multiple, redundant receptors within the nine-exon subfamily tuned specifically for this critical compound.

Robust responses to CHC extracts and a panel of hydrocarbons found in *H. saltator* were observed among the majority of nine-exon *HsOr*s tested in a parallel study although only one (*HsOr*259-L2) of the two nine-exon receptors that were in our *HsOr* panel responded strongly to a CHC (to C37). In contrast, several of the other 23 *HsOr*s examined in this study, representing a diverse range of the other OR subfamilies of HsORs, also display significant responses to these CHC-associated ligands. Although responses to volatile nonhydrocarbon general odorants were also sparse and well-distributed phylogenetically across all of the OR subfamilies tested including the nine-exon ORs, they nevertheless encompassed different receptors from the ones that responded robustly to hydrocarbons.

In light of their complex phylogenetic structure and the sheer number of uncharacterized *HsOr*s, it is difficult to draw firm conclusions, but it nevertheless seems reasonable that absolute and inviolate odor-coding boundaries for ant OR subfamilies in relation to pheromonal and nonpheromonal stimuli do not exist. These questions are further complicated by the likelihood that additional membrane proteins and other factors may be required in order for pheromone ligands to elicit responses, as has been observed with the *Drosophila* pheromone receptor Or67d (Benton 2007). The discriminatory power afforded by the combinatorial interactions of the large numbers of ant ORs, acting in concert with other chemosensory components, most notably the IR and GR gene families, seems more than capable of addressing the extraordinary challenges associated with the complex chemical ecology of eusocial colonies. That said, by analyzing members of distinct subfamilies of *HsOr*s beyond the highly expanded nine-exon subfamily and those with differential abundance among castes and genders, this study represents a quantum advance in the study of the molecular genetics of these critical peripheral chemoreceptors that are responsible for initiating many, if not all, of the distinct social behaviors that are the hallmark of these eusocial insects.

Materials and Methods

Odorant Receptor Cloning.

Full-length *HsOr* genes were subcloned or commercially synthesized (Genscript) for transgenic expression of *HsOr* genes in flies by insertion into a preexisting insertion site in the

Drosophila genome, using the phiC31 integrase recombination system (Groth et al. 2004). See Appendix B for full details.

Drosophila Genetics.

For SSR and EAG experiments, experimental *D. melanogaster* genotypes were either w^{1118}*;* w^{+}, *UAS-HsOr;* w^{+}, *Orco-GAL4* or w^{1118}; *+ ;* w^{+}, *UAS-HsOr/*w^{+}, *Orco-GAL4*. Control flies were w^{1118}*; +;* w^{+}, *Orco-GAL4*.

Eletrophysiology.

Flies were tested 2–10 d after eclosion for both single-sensillum and whole antennal EAG recordings, with an n = 4–8 per *UAS-HsOrX* line. We then manually normalized those responses to the Orco-GAL4 control. For SSRs, the ab2 sensillum-type was used for all recordings. Each compound (Table A-3) was dissolved in pentane and 20 nmol of the compound was applied to each delivery cartridge. The cartridges were then heated for 1 s with a handheld butane torch, and then air was puffed through the heated cartridge into an airstream, and over the fly antenna for a 500-ms duration, using 3 mL of humidified air. See Appendix B for additional details.

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Author Contributions

J.D.S., S.T.F., A.R., and L.J.Z. designed research; J.D.S. and S.T.F. performed research; J.G.M. and J.L. contributed new reagents/analytic tools; J.D.S., G.M.P., S.T.F., A.R., and L.J.Z. analyzed data; J.D.S., G.M.P., S.T.F., J.G.M., J.L., A.R., and L.J.Z. wrote the paper; and S.L.B.,

D.R., J.L., A.R., and L.J.Z. contributed to overall project design and direction from the inception of this work.

Chapter III. Odor coding of nestmate recognition in the eusocial ant *Camponotus floridanus*[‡]

Abstract

In eusocial ants, aggressive behaviors require the ability to discriminate between chemical signatures such as cuticular hydrocarbons that distinguish nestmate friends from non-nestmate foes. It has been suggested that a mismatch between a chemical signature (label) and the internal, neuronal representation of the colony odor (template) leads to aggression between non-nestmates. Moreover, a definitive demonstration that odorant receptors are responsible for the processing of the chemical signals that regulate nestmate recognition has thus far been lacking. To address these issues, we have developed an aggression-based bioassay incorporating highly selective modulators that target odorant receptor functionality to characterize their role in nestmate recognition in the formicine ant *Camponotus floridanus*. Electrophysiological studies were used to show that exposure to either a volatilized antagonist or an agonist eliminated or dramatically altered signaling, respectively. Administration of these compounds to adult workers significantly reduced aggression between non-nestmates without altering aggression levels between nestmates. These studies provide direct evidence that odorant receptors are indeed necessary and sufficient for mediating aggression towards non-nestmates. Furthermore, our observations support a hypothesis in which rejection of non-nestmates depends on the precise decoding of chemical signatures present on non-nestmates as opposed to the absence of any information or the active acceptance of familiar signatures.

Summary Statement

Broad inhibition as well as activation of peripheral odorant receptor signaling decreases aggression between non-nestmate ants consistent with a "lock-and-key" model that requires OR-based detection of unambiguous non-nestmate chemical labels.

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Introduction

Aggression comprises a range of important social interactions with implications for individual behavior as well as the collective integrity of animal societies. While hostile behaviors can be observed throughout the Metazoa (Blanchard and Blanchard 1977; Hölldobler and Wilson 1990; Ayre and Grosberg 1995; Mitani, Watts, and Amsler 2010; Scheel, Godfrey-Smith, and Lawrence 2016), recently established experimentally tractable eusocial insect models present an opportunity to investigate the mechanistic basis of aggression within a social context. In this regard, ants provide a compelling model for the study of aggression and its triggering mechanisms. Ant colonial lifestyles and reproductive hierarchies are maintained by aggressive social interactions that are modulated by their ability to detect, discriminate between, and respond to a large array of chemical cues (Hölldobler and Wilson 1990; Morel, Vandermeer, and Lavine 1988; Endler et al. 2004; Moore and Liebig 2010). Moreover, recent studies (Yan et al. 2017; Trible et al. 2017) have demonstrated the value of applying novel genetic and molecular techniques that have restricted availability in the study of humans and other social primates.

The formicine ant *Camponotus floridanus* live in colonies that are founded by a single reproductive queen (Hölldobler and Wilson 1990; Gadau et al. 1996). Workers nurse the queen's offspring, forage for food, and defend nest and territory from non-nestmates (nNMs) (Hölldobler and Wilson 1990). Although individual workers contribute to broader colony-level phenotypes, the integrity of social behaviors depends on the collective actions of the colony (Gordon 2015). Among these social behaviors, nestmate (NM) recognition is especially important for establishing and maintaining discrete societal boundaries for *C. floridanus* and many other species of ant (Hölldobler and Wilson 1990). NM recognition is a dynamic behavior that has been suggested to occur when an individual ant compares chemically encoded "labels" that it encounters with potentially multiple neural-encoded "templates" that represent its own particular global colony chemosensory signature whereby a mismatch between a foreign label and the recognition templates leads to aggression between nNMs (Neupert et al. 2018; Vander Meer and Morel 1998; Obin and Vandermeer 1989). The foreign label is derived, at least in part, from subtle variations in the profile of cuticular hydrocarbons (CHCs) that distinguish nNMs from NMs (Morel, Vandermeer, and Lavine 1988; Guerrieri et al. 2009; Neupert et al. 2018).

Early genetic models provided a framework for understanding the criteria required to assess colony membership status when comparing the recognition template to a respective label

(Crozier and Dix 1979). These have been broadly organized into two categories: the gestalt model, in which label sharing between individuals yields a distinct template based on a blend; and individualistic models, which include requiring the exact matching of the label to the template ("genotype matching"), rejection of any labels containing cues not found in the template ("foreign-label rejection"), and the acceptance of labels that overlap with the template ("habituated-label acceptance"). Similarly, there have been efforts to elucidate the rules governing template-label matching within a phenotypic context (Guerrieri et al. 2009; Neupert et al. 2018; Sherman, Reeve, and Pfennig 1997). These models suggest that ants discriminate between friends and foes based on the presence and/or absence of NM ("desirable") cues or nNM ("undesirable") cues. While it was initially proposed that ants accept individuals if they possess desirable cues (D-present) or if they lack undesirable cues (U-absent) to the exclusion of all others (Sherman, Reeve, and Pfennig 1997), more recent evidence suggests that ants actively detect foes but not friends through the detection of nNM odor cues (simple U-present model) (Guerrieri et al. 2009). Importantly however, discrimination may also occur when critical components of the CHC profile are missing (Neupert et al. 2018). These studies suggest that multiple templates are used to assess different labels, and that the importance of a given component of the label varies.

While the importance of CHCs in mediating NM recognition among ants is well established, several alternative hypotheses have been proposed for the neuronal and molecular mechanisms allowing ants to distinguish friends from foes (Ozaki et al. 2005; Guerrieri et al. 2009; Brandstaetter AS 2011; Neupert et al. 2018; Brandstaetter 2011; Sherman, Reeve, and Pfennig 1997; Crozier and Dix 1979). In all of these models, CHCs and other semiochemicals are initially detected by the peripheral olfactory sensory system which relies on three major classes of peripheral chemosensory receptors—odorant receptors (ORs), gustatory receptors, and ionotropic receptors. Insect ORs are expressed in olfactory receptor neurons (ORNs) housed within sensilla on the antennae (reviewed in (Suh, Bohbot, and Zwiebel 2014)), where they function as heteromeric complexes consisting of an obligate and conserved OR co-receptor (Orco) and at least one "tuning" OR that determines odorant (ligand) specificity (Zhou et al. 2012; Larsson et al. 2004; Benton et al. 2006; Sato et al. 2008; Wicher et al. 2008; Jones et al. 2011; Pask et al. 2011). Several studies have revealed a large expansion of the *OR* gene family in ants and other eusocial insects (Zhou et al. 2012; Zhou et al. 2015; Smith, Smith, et al. 2011; Robertson and Wanner 2006; Bonasio et al. 2010; Wurm et al. 2011; Oxley et al. 2014; Werren et al. 2010; Smith, Zimin, et al.

2011). Members of this chemoreceptor family detect socially relevant chemical cues such as CHCs (Slone et al. 2017; Pask et al. 2017).

Despite the long-held appreciation for the role of CHCs and other chemical cues in mediating NM recognition and social behaviors in ants, little is known about the specific molecular components of olfactory signal transduction that are active in regulating NM recognition and the triggering of aggression toward nNMs. Electrophysiological studies of *Camponotus japonicus* first suggested that a dedicated multiporous NM recognition sensilla exhibited an all-or-none response to nNM CHC blends but, importantly, did not respond to NM CHC blends—thus leading to a model in which ants are desensitized and ultimately anosmic to their own odor cues (Ozaki et al. 2005). In contrast, recent studies using both antennal electrophysiology and antennal lobe calcium imaging in the related ant species *C. floridanus* demonstrate these ants are capable of detecting both nNM and NM odors (Brandstaetter AS 2011; Brandstaetter 2011; Sharma et al. 2015). It has been proposed that these seemingly contradictory findings support a model in which two sensilla subtypes—one broadly tuned to hydrocarbons and the other tuned to specific hydrocarbons—facilitate habituation to different labels (Bos and d'Ettorre 2012).

The paucity of data in this regard may be attributed, at least in part, to the challenges of targeted molecular approaches currently available in the study of Hymenopteran insects. The development of these techniques represents an important step towards understanding the function and evolution of the molecular mechanisms involved in complex social behaviors such as NM recognition with the potential to shed light on longstanding questions within the field of social insect biology. To begin to address this, a series of behavioral, physiological, and gene knockout studies were carried out to characterize the relationship between ant ORs and CHCs as well as other biologically salient chemical cues. These studies demonstrated that CHCs and other general odorants were broadly detected across the various OR subclades while CRISPR-mediated gene knockout of *orco* resulted in alterations of both solitary and social behaviors as well as profound neuroanatomical disruptions in the antennal lobe (Slone et al. 2017; Pask et al. 2017; Yan et al. 2017; Trible et al. 2017). Taken together, these studies suggest that ORs play a critical role not only in a diversity of behaviors but also importantly in ant neural development.

We now extend these studies by employing a set of highly specific Orco allosteric modulators to examine the role of OR signaling in mediating NM recognition. The first member of this unique class of pharmacological agents (known as VUAA-class actives) was identified

through high-throughput screening for small molecule modulators of mosquito Orco/OR complexes expressed in HEK293 cells (Jones et al. 2011; Pask et al. 2011; Rinker et al. 2012). In subsequent studies that revealed extraordinarily narrow structure-activity relationships, several additional VUAA-class actives were identified and characterized that now comprise several more potent agonists (including VUAA4 used here), a non-competitive antagonist (VUANT1, used here) as well as an inactive structural analog (VUAA0, used here) (Jones et al. 2011; Jones et al. 2012; Rinker et al. 2012; Taylor et al. 2012; Romaine et al. 2014). The selective potency of these modulators against Orco targets in both volatile and non-volatile forms is conserved across a wide range of insect orders (Jones et al. 2012; Tsitoura and Iatrou 2016; Tsitoura, Koussis, and Iatrou 2015; Hansen et al. 2014; Sharma et al. 2015). Indeed, VUAA-Orco interactions have recently been directly confirmed by cryo-electron microscopy studies characterizing the structure of an Orco tetramer from the parasitic fig wasp *Apocrypta bakeri* (Butterwick et al. 2018). Importantly, single-sensillum recordings of the female-specific basiconic sensilla in *C. floridanus* have demonstrated the potentcy of at least one of these VUAA-class actives, such that exposure to VUANT1 significantly reduced olfactory responses to both a blend of hydrocarbons and cuticle extract (Sharma et al. 2015).

The use of these unique and highly specific chemical tools allowed us to selectively target Orco and therefore the functionality of all OR/Orco complexes to examine NM recognition with altered OR signaling in wild-type adult *C. floridanus* workers. This was an essential aspect of our approach in light of the broad neuroanatomical alterations that have recently been observed in the development of the antennal lobes of Orco mutants in two ant species (Trible et al. 2017; Yan et al. 2017) which are reasonably likely to impact olfactory processing and behavior. Indeed, the use of volatile Orco modulators represent a novel and requisite approach for disrupting OR functionality in insects such as ants that require alternatives to CRISPR-mediated targeting of pleiotropic genes such as *orco* (Trible et al. 2017; Yan et al. 2017). Here, we report studies that specifically address the odor coding of NM recognition by utilizing a novel volatilization paradigm. In this manner, we are able to directly test the hypotheses that aggression is triggered by the active detection and decoding of discrete chemosensory stimuli and more specifically that the functionality of the OR-Orco ion channel complex is necessary for NM recognition.

Materials and Methods

Ant Husbandry

Nine distinct laboratory colonies of *Camponotus floridanus* originating from field collections generously obtained by Dr. J. Liebig (Arizona State University) from the Long Key (D242) and Sugarloaf Key (D601) and Dr. S. Berger (University of Pennsylvania) from the Fiesta Key (C6, K17, K19, K28, K31, K34, and K39) in South Florida, USA. All colonies were independently maintained at 25°C, ambient humidity (approximately 70%), with a 12-h light:12-h dark photoperiod. Each colony was provided with Bhatkar diet, crickets, 10% sucrose solution, and distilled water three times per week. Adult minor workers were used for all experiments and were sampled from throughout the colonies.

Ablation Aggression Bioassay

Tests were conducted during the ZT diel light cycle between ZT2 and ZT12 at ambient room temperature and humidity and performed using a six-well culture plate with polytetrafluoroethylene-coated well walls (DuPont®). Individual wells of the six-well culture plate served as distinct bioassay arenas for behavioral trials (Figure C-1). In preparation for experiments, each well (9.6cm^2) of the six-well culture plate was fitted with a removable plastic divider that partitioned the well into two halves. The six-well culture plate and dividers were sterilized using ethanol, air dried, and positioned on top of a light box. Each individual bioassay well utilized two adult minor ants that were selected from either the same home colony (NMs) or two distinct colonies (nNMs). All ants were handled wearing gloves and using sterile, soft-tipped metal forceps and were subsequently discarded in the freezer (-20˚C) after each bioassay to ensure each ant was used only once.

Subject ants were briefly anesthetized with CO_2 before removing their antennal flagella via an incision across the distal portion of the scape using a clean, unused razor blade. Bilaterally ablated ants had both flagella removed while unilaterally ablated ants had only a single (right or left, randomly selected) flagellum removed. Sham treated ants were anesthetized with CO_2, and the razor was gently touched to the antennae without damaging any structures. Subsequent to ablation (or sham) treatment, ants were allowed to recover along with similarly treated NMs for at least 2 hours prior to testing.

Prior to bioassays, two ants (NMs or nNMs) were placed into each well arena, one in either half, and allowed 10 min to acclimate to handling. To document normal ant behavior within each well arena, mobility was recorded using a digital high definition camera (Panasonic® HC-V750) for 3 min (detailed below). The plastic divider within each well arena was subsequently removed and all ant interactions again recorded for 3 min. The order in which the treatments were conducted as well as the colony the ants were selected from for any given trial were randomized using RANDOM.ORG (Randomness and Integrity Services Ltd.).

Electroantennography

Electroantennograms were performed using an IDAC-232 (Ockenfels Syntech GmbH, Germany) controller linked to a Windows XP computer running EAG2000 (Ockenfels Syntech GmbH, Germany) software. A set of 12x75mm test tubes placed atop a heat block set at 260°C containing 0.025g of the respective treatment compound (VUAA0, VUANT1, or VUAA4) or an empty tube (blank control) were connected to a Syntech CS-05 Stimulus flow Controller (flow rate of 1.5cm^3/s) (Ockenfels Syntech GmbH, Germany). Using this setup, both the constant background airflow as well as the 500-ms pulse of stimulus compound contained volatilized VU-class compounds or heated air (in the case of the blank control).

Subject ants were placed in a 20µL disposable pipet tip that was modified such that the tip opening was sufficiently wide to allow the unimpeded exposure of the head and antennae. To prevent movement of the preparation which might otherwise reduce the signal-to-noise of the recordings, the head and mandibles of the ant were restricted with wax. Borosilicate glass capillaries (FIL O.D.:1.0mm, World Precision Instruments, Inc.) were customized for EAGs on a P-2000 laser micro-pipette puller (Sutter Instruments), backfilled with 10^{-1} M KCl and 0.05% PVP buffer and placed over tungsten electrodes. A 30-guage needle was used to puncture the right eye to allow for insertion of the reference electrode. The recording electrode was placed over the distal tip of the left antenna. Decane (C10) (CAS: 124-18-5, Sigma-Aldrich) was serially diluted in hexane (0.1 µg/µl, 1 µg/µl, 10 µg/µl, 20 µg/µl, and 200 µg/µl). An odor cartridge was filled with 10µl of decane solution (or hexane alone as a solvent control) and a handheld butane torch (BernzOmatic, Worthington Industries) was used to volatilize the decane compound by heating the odor cartridge for 1.5 seconds. 4-methyl-3-heptanol (4M3H) (CAS: 14979-39-6, Sima-Aldrich) was serially diluted in paraffin oil (10^{-5} M, 10^{-4} M, 10^{-3} M, 10^{-2} M). Serial concentrations

were assayed sequentially starting with the lowest concentration and ending with the highest concentration. Decane responses were normalized to the hexane solvent control (set at 0) and 4M3H responses were normalized to the paraffin oil solvent control (set to 0) to account for changes in sensitivity and/or antennae degradation over time throughout the assay, and these values were used for subsequent data analysis.

Volatile Orco Modulator Aggression Bioassay

To facilitate the administration of a continuous flow of air containing volatilized VUAA-class compounds (all custom synthesized as dry solids in-house at Vanderbilt University (Jones et al. 2011; Jones et al. 2012; Taylor et al. 2012; Romaine et al. 2014)) into the aggression arena, bioassays were conducted in arenas consisting of modified square plastic boxes with a total area of 85cm^2 (Pioneer Plastics Inc. ®) (Figure C-1). Mirroring the electroantennography, conditioned air (78% Nitrogen, 21% Oxygen) was delivered at a constant 34kpa from a compressed source (Nashville Gas LLC) to the test arena at a flow rate of approximately 50cm^3/s. Air was controlled by a dual Y valve affixed to the compressed air tank and delivered through a 12x75mm test tube atop a heat block set at 260°C which contained 0.025g of the respective treatment compound (VUAA0, VUANT1, or VUAA4) or an empty tube (Blank control) via 18G needles inserted into a rubber septum affixed to the top of the test tube. Air was cleared from the arena through a dedicated exhaust system. Trials were recorded using a digital high definition camera and scored as described below. Although two plastic tubes were affixed to the arena during the volatilization aggression bioassays, only a single tube was actively delivering the test compound or heated air control (Figure C-2). In each assay, ants were acclimatized underneath 35mm Petri dish lids (prewashed with ethanol) for 10 minutes after which the lids were then removed (allowing the ants to interact), the airflow started, and the ants' behavior was then recorded for the 3-minute test period. All treatment compounds were randomized and coded independently such that the investigator was blind to the treatment identity. Furthermore, the sequential order in which the compounds were tested as well as the colony from which the ants were selected for any given trial was randomized using RANDOM.ORG (Randomness and Integrity Services Ltd.).

Aggression Bioassay Scoring

Digital video recordings of all bioassays were viewed post hoc and aggression incidents manually scored for analyses. Trials in which ants did not interact (N=23), were disrupted physically during removal of the plastic barrier (N=5), or appeared injured/unconscious at trial onset (N=3) were discarded from further analyses along with their respective mobility controls in the case of the antennal ablation bioassays. These interactions were scored by three independent, blind observers in 10 s intervals using a binary scale such that aggression either did or did not occur (a score of 1 or 0, respectively). Prior to scoring, each observer was trained to recognize "aggression" as instances in which one or both ants were lunging, biting, or dragging one another. Each 10 s time interval was scored as either containing an instance of aggression or not to establish the proportion of time the ants were engaged in aggressive behavior. An aggression index was calculated by dividing the number of observed acts of aggression by the total number of observed time intervals. The mean aggression index of each video recording across all three independent scores was used for subsequent statistical analysis.

Mobility Control Parameters

Mobility control videos were analyzed using an automated tracking software package (Ethovision® XT v8.5, Noldus Information Technology) to calculate total distance traveled (cm), percentage of time spent moving (%), and the frequency of rotations (count). Time spent moving/not moving was calculated with thresholds of 0.30cm/s (start velocity) and 0.20cm/s (stop velocity) as determined by the EthoVision® XT software with an averaging interval of 1 sample. A single rotation was defined as a cumulative turn angle of 90° over a distance of 1.00cm. Turns in the opposite direction of less than 45° were ignored. The sum of both clockwise and counterclockwise rotations was used to determine rotational frequency. Trials in which the subject ant was not found for at least 95% of the recording were discarded (N=15).

Mechanically Evoked Biting and Mandible Opening Response (BMOR) Bioassay

To determine whether disrupting Orco-mediated olfactory signaling disrupted broadly aggression in a non-social context, individual adult minor workers were briefly anesthetized with CO_2 before being secured with wax in a modified 200µl pipette tip such that the head and antennae were accessible. The ants were allowed to acclimate for 10 minutes before being exposed to a

continuous flow of heated air alone or volatilized VU-class compounds as described above in the Volatile Orco Modulator Aggression Bioassays. A clean, ethanol washed 3.61/0.4g Von Frey hair filament (Baseline® Fold-Up™ Monofilaments Item #12-1741) was then gently brushed along the anterior portion of the ant's head from the ventral to the dorsal side five times. Aggression was scored by six independent, blinded observers on a binary scale such that biting or attempting to bite the filament or wide opening of the mandibles (i.e. the mandibles were opened beyond parallel) either did (score of 1) or did not (score of 0) occur during the duration of the trial. An aggression index was calculated by taking the average score across all observers and used for subsequent statistical analysis. Trials in which the ants did not recover from the CO_2 treatment were discarded.

Statistical Analysis

Statistical analyses were performed using Graphpad Prism v8.0.0 (GraphPad Software, Inc). For the aggression bioassays, a two-way ANOVA was first performed followed by Holm-Sidak's multiple comparisons test to compare NM vs. nNM aggression as well as aggression across antennal treatments. For the antennal ablation mobility controls as well as the BMOR bioassays, a Kruskal-Wallis test was performed followed by Dunn's correction for multiple comparisons. As the volatilization mobility controls had matched samples across different time points, a repeated measures two-way ANOVA with the Geisser-Greenhouse correction for violations of sphericity was performed. For the electroantennography, linear regression analysis was used to test whether the best-fit slope differed significantly from 0 (i.e. a straight line with no dose response). The response to the solvent control (i.e. 0 µg/µl of decane or 0 M of 4M3H) was normalized to 0mV, therefore the Y-intercept was constrained to X=0, Y=0. The inclusion and exclusion criteria for all samples was pre-established. The number of replicates for each study were as follows: Ablation Aggression Bioassays (6-10); Mobility Controls (Ablation) (24-29); Volatile Orco Modulator Aggression Bioassays (10-12); Volatile Orco Modulator BMOR Bioassay (10-11); Mobility Controls (Volatilization) (7-9); Electroantennography (5-6). Information regarding the statistical test performed and the results from these analyses have been detailed in Table C-1.

List of Abbreviations

Nestmate (NM), non-nestmate (nNM), cuticular hydrocarbon (CHC), odorant receptor (OR), odorant receptor co-receptor (Orco), odorant receptor neuron (ORN).

Results

Nestmate Recognition Requires Antennal-based Signaling

The initial phase of this study was to develop an olfactory-based NM recognition bioassay in which two ants—NMs from the same home colony or nNMs from two different colonies—were able to interact with one another after an acclimation period (Figure III-1A). To this end, we initially took a broad approach to assess the role of olfactory signaling in modulating NM/nNM aggression in the context of pairwise trials conducted using adult *C. floridanus* minor worker ants with either unilateral or bilateral antennal ablations. As it has been long established that antennal ablation is expected to decrease aggression between nNMs (Forel 1928; Wang et al. 2016), these assays were undertaken to validate our experimental design. In these studies, both control *C. floridanus* workers ($t=4.404$, $P=0.0001$) as well as those having undergone unilateral ablations ($t=5.438$, $P<0.0001$) were able to routinely discriminate nNMs from NMs and display only nNM aggression (Two-Way ANOVA with Holm-Sidak's Multiple Comparisons Test) (Figure III-1B, Table C-1). In contrast, ants with bilateral antennal ablations displayed a significant and indeed near-complete reduction in aggression against nNMs ($t=3.384$, $P=0.003$). These data are consistent with the widely reported ability of *C. floridanus* workers to robustly discriminate between nNMs and NMs and supports the hypothesis that their chemosensory apparatus is required to recognize and trigger aggression against nNMs (Hölldobler and Wilson 1990; Morel, Vandermeer, and Lavine 1988; Leonhardt, Brandstaetter, and Kleineidam 2007; Guerrieri et al. 2009; Ozaki et al. 2005; Brandstaetter AS 2011; Slone et al. 2017; Pask et al. 2017; Neupert et al. 2018; Forel 1928; Wang et al. 2016).

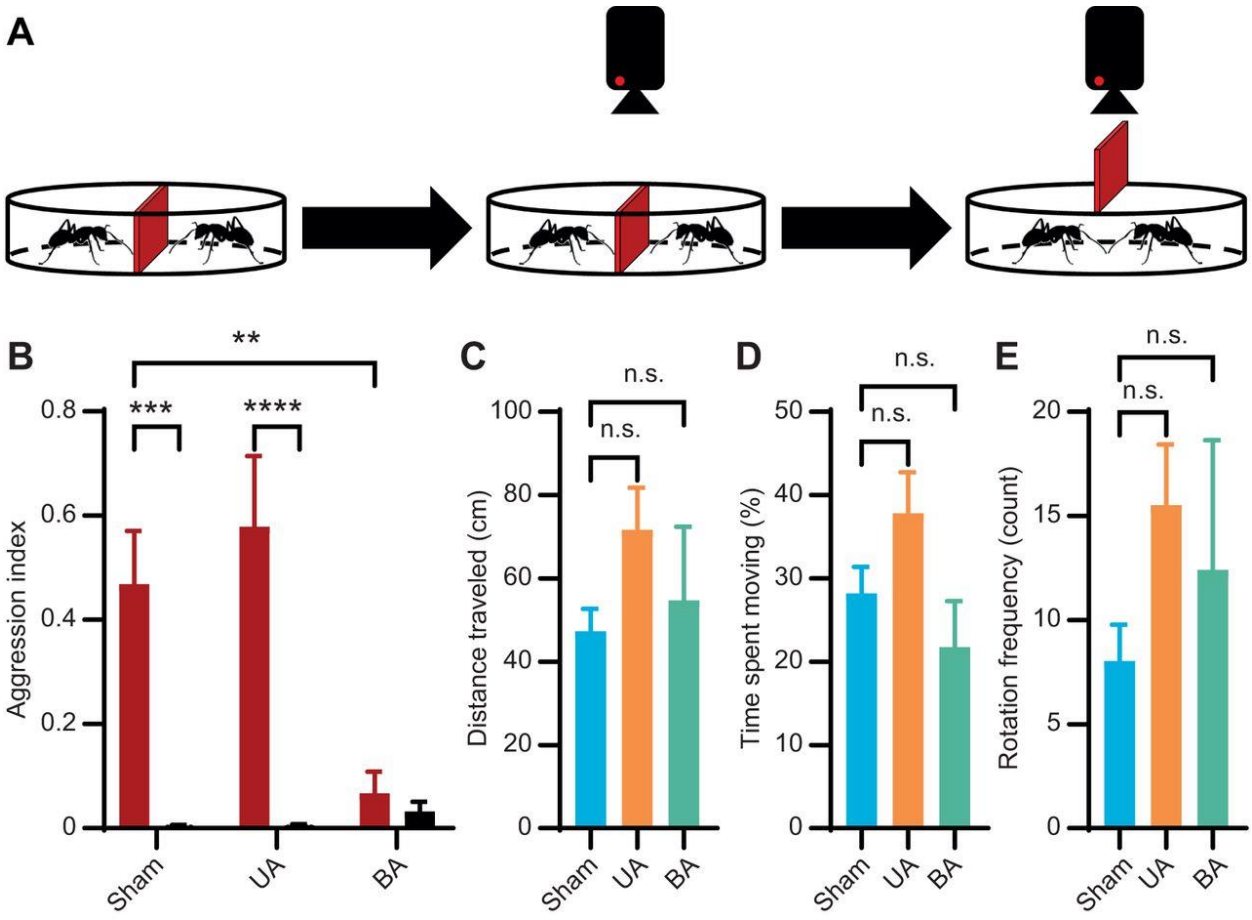

Figure III-1. Aggression and mobility responses of adult minor workers following antennal ablation.
Sham, control; UA, unilateral ablation; BA, bilateral ablation. (A) Schematic representation of the ablation bioassay depicting the acclimation period (left), mobility controls (center) and aggression bioassay (right). (B) Bilateral antennal ablation significantly reduced non-nestmate (nNM, red) aggression compared with the sham control. Black, nestmates (NMs). Two-way ANOVA with Holm–Šidák's multiple comparisons test (biological replicates: sham NMs N=9, sham nNMs N=10, UA NMs N=10, UA nNMs N=9, BA NMs N=6, BA nNMs N=6). (C–E) There was no significant difference in mobility between the sham control and the ablation treatments, as assessed by distance traveled (C), time spent moving (D) and rotation frequency (E). Kruskal–Wallis test (biological replicates: sham and UA N=29, BA N=24). Bars display mean; error bars display s.e.m. Asterisks indicate significance: **P<0.01, ***P<0.001, ****P<0.0001. n.s., not significant.

To further control for potentially confounding variables—including the outright death or incapacitation of the ants due to the damage sustained from the ablations—we measured a number of other behavioral indicators including total distance traveled, percentage of time spent moving/not moving, and the frequency of rotations using an automated tracking program (see Methods). Here, the activity of a single ant was recorded for three minutes immediately following the 10-minute acclimation period and preceding the ablation aggression bioassays (Figure III-1A). These assays revealed no significant difference between the sham control and the ablation treatments (Figure III-1C-E, Table C-1). Treated ants were able to recover from the injury and

retain fundamental aspects of mobility, and unilaterally ablated workers kept the ability to discriminate between NMs and nNMs. This suggests that the decrease in aggression was likely due to the absence of antennae-mediated signaling as opposed to confounding variables introduced by the ablation treatment. However, as the removal of the antennae disrupts a broad range of both mechanoreceptors as well as chemoreceptors (Nakanishi et al. 2009), a more targeted approach was required to assess the specific function of OR-dependent chemoreceptor signaling in this context.

Nestmate Recognition is an Active, OR-dependent Process

In order to further examine this process within the narrow context of assessing the role of ORs in NM recognition and aggression, we adapted our bioassay to incorporate the sustained volatile administration of a set of highly specific Orco allosteric modulators (Figure III-2A, Figure C-1). While the use of certain VUAA-class actives has already been shown to disrupt OR-mediated detection of a blend of hydrocarbons (C7-C40) and cuticle extract (Sharma et al. 2015), we sought to validate the efficacy of these compounds in the context of our experimental setup where they were delivered within a constant background airflow to our aggression bioassay arena. We performed electroantennograms (EAGs) to assess whole antennal responses to several concentrations of the hydrocarbon decane (C10) as well as 4-methyl-3-heptanol (4M3H) in adult workers exposed to heated air (blank control) or volatilized compound (Figure III-2A). These two compounds were chosen because they elicited strong responses in the basiconic sensilla which presumably contains many OR-expressing neurons (Sharma et al. 2015; McKenzie et al. 2016).

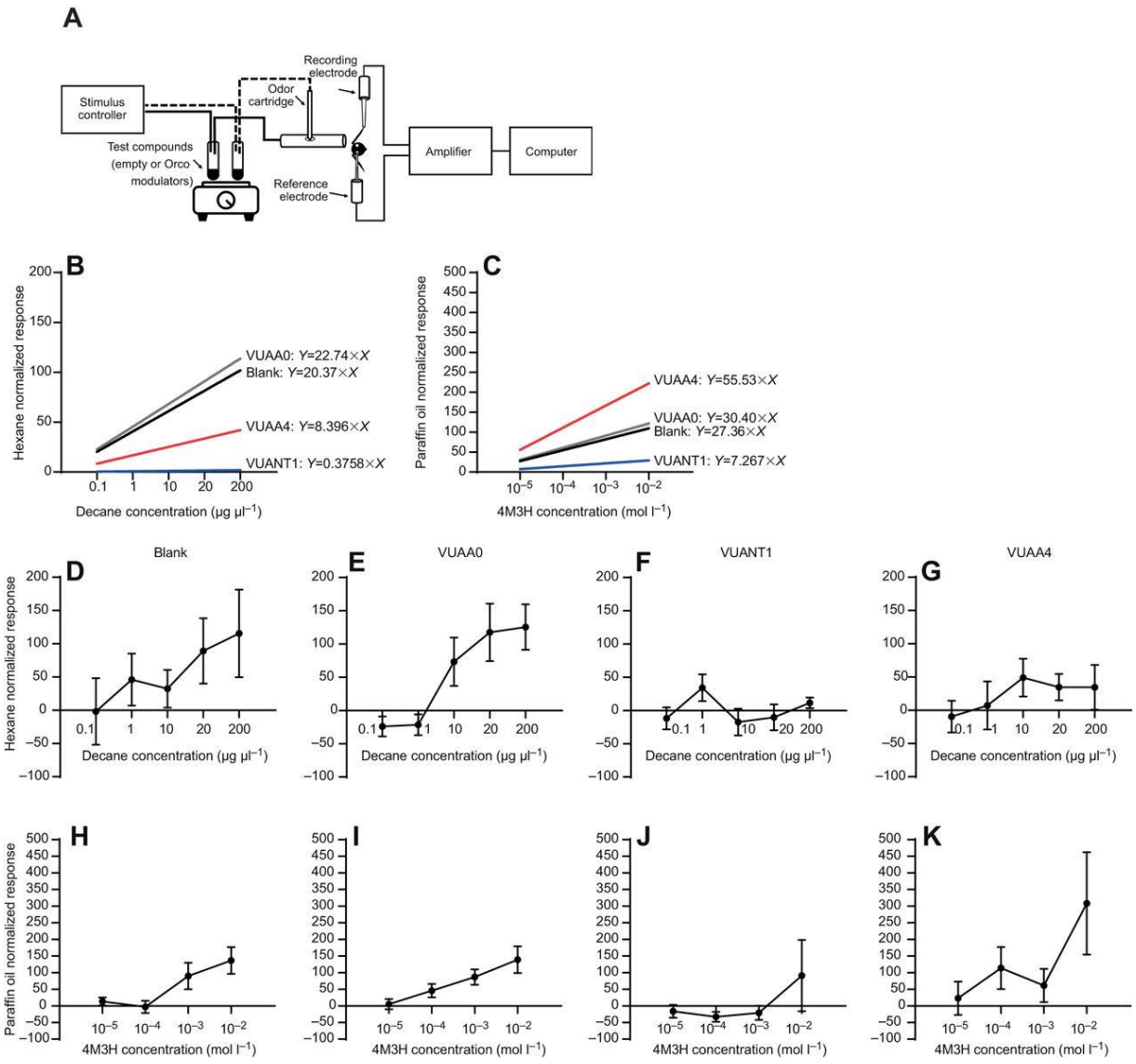

Figure III-2. Electrophysiological responses of adult minor workers to decane or 4M3H under different background airflow conditions.

(A) Schematic diagram of the electroantennogram apparatus. (B,C) Best-fit lines derived from the solvent (hexane or paraffin oil) normalized responses to serial concentrations of decane (D–G) or 4M3H (H–K), respectively, for blank (control, heated air alone), VUAA0 (inert chemical analog control), VUANT1 (Orco antagonist) and VUAA4 (Orco agonist) backgrounds. The slope of the best-fit line for blank, VUAA0 and VUAA4 for both decane and 4M3H was significantly different from 0. Linear regression (biological replicates: decane blank *N*=5, VUAA0 *N*=5, VUANT1 *N*=6, VUAA4 *N*=5; 4M3H blank *N*=6, VUAA0 *N*=6, VUANT1 *N*=5, VUAA4 *N*=5; see Table C-1). Points display mean; error bars display s.e.m.

For decane, we observed similar dose-dependent responses in both our blank control and VUAA0 (Figure III-2B and D-E). Indeed, linear regression analysis revealed that the slope of the blank control ($F(1, 24)=11.39$, $P=0.0025$) and VUAA0 ($F(1, 24)=25.31$, $P<0.0001$) are significantly different from 0 (i.e. a flat line) (Figure III-2B, Table C-1). Consistent with

expectations, the slope of VUANT1 was not significantly different from 0 (Figure III-2B and F, Figure C-1), suggesting that exposure to this compound completely eliminated dose-dependent detection of decane. Volatile administration of VUAA4 also disrupted hydrocarbon detection, however it did not eliminate OR signaling. Rather, it displayed a muted and partially dose-dependent response with seemingly static, yet low, responsiveness at higher concentrations (Figure III-2B and G). These are likely the result of broad ORN desensitization after prolonged exposure to this potent Orco agonist. Nevertheless, the slope of VUAA4 was significantly different from 0 ($F(1, 24)=0.0320$, $P=0.032$) (Figure III-2B, Table C-1), suggesting that dose-dependent hydrocarbon detection and ORN firing still occur albeit not in the same manner as the controls. With regard to 4M3H, we again observed similar dose-dependent responses in the blank control ($F(1, 23)=22.58$, $P<0.0001$) and VUAA0 ($F(1, 23)=42.11$, $P<0.0001$), and these responses were eliminated in the VUANT1 treatment (Figure III-2C and H-J). Responses to VUAA4, however, were substantially increased (Figure III-2C and K). These elevated responses are consistent with the expected role of VUAA4 as an Orco agonist. These observations highlight the profound effects that acute volatile administration of VUAA4 has on olfactory signaling. Taken together, these data foster the view that ambiguous/altered odor coding results from a combination of both cryptic activation and desensitization of ORNs. Furthermore, responses to odorants are not completely eliminated but nevertheless deviate from control responses. Altogether, these studies demonstrate that VUAA-class actives disrupt Orco-mediated olfactory signal transduction in ants.

Using this newly established volatilization paradigm, we then sought to determine the precise role of OR-signaling in mediating aggression towards nNMs. Ants taken from across nine independent colonies exposed to either Orco modulator displayed a significant reduction, and indeed a near complete elimination, of aggression towards nNMs (Two-Way ANOVA with Holm-Sidak's Multiple Comparisons Test; VUANT1 – $t=2.372$, $P=0.0399$; VUAA4 – $t=3.466$, $P=0.0026$) (Figure III-3A, Table C-1). Importantly, ants treated with either the Orco agonist or the antagonist displayed no significant difference in their responses to NMs. This lack of misdirected aggression toward NMs as well as the failure to correctly attack nNMs in ants treated with these highly selective Orco/OR modulators demonstrated that, in *C. floridanus*, aggression is specifically mediated by the OR-dependent detection of specific and unambiguous odor cue signatures from nNM foes rather than the general absence or incorrect processing of familiar signatures of NM friends.

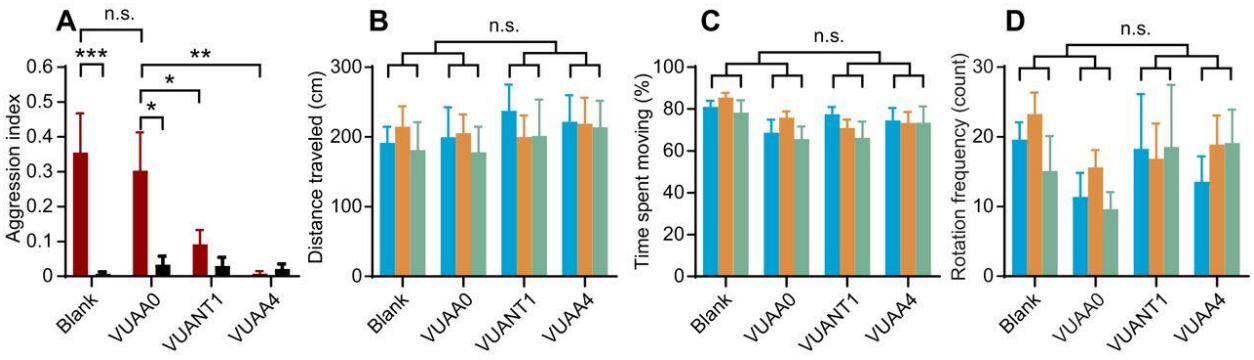

Figure III-3. Aggression and mobility responses of adult minor workers during exposure to volatilization treatments.

(A) Disrupting Orco-mediated olfactory signal transduction significantly reduced aggression towards nNMs (red). Black, NMs. Two-way ANOVA with Holm–Šidák's multiple comparisons test (biological replicates: blank NMs *N*=10, nNMs *N*=12; VUAA0 NMs *N*=10, nNMs *N*=11; VUANT1 NMs *N*=12, nNMs *N*=10; VUAA4 NMs *N*=10, nNMs *N*=12). (B–D) There was no significant interaction between treatments across the mobility parameters tested (acclimation, blue; treatment, yellow; recovery, green). RM two-way ANOVA with Greenhouse–Geisser epsilon (biological replicates: blank *N*=8, VUAA0 *N*=8, VUANT1 *N*=7, VUAA4 *N*=9). Bars display mean; error bars display s.e.m. Asterisks indicate significance: **P*<0.05, ***P*<0.01, ****P*<0.001. n.s., not significant.

Furthermore, in order to assess whether the disruption of OR-signaling reduced aggression within the narrow social context of NM recognition or alternatively acted to broadly inhibit aggressive behaviors, we conducted parallel bioassays that utilized mechanical rather than chemical stimuli to evoke aggression. Using a modified aggression bioassay based on previous methods described in (Guerrieri and d'Ettorre 2008) and (Gospocic et al. 2017), individual ants were challenged with a chemically neutral mechanical stimulus (i.e. a clean Von Frey filament) and subsequently scored for biting responses as well as wide opening of the mandibles as indicators of aggression. Importantly, as there was no significant difference in aggression among the various treatment groups (Figure C-2), we could conclude that disrupting Orco-mediated olfactory signaling did not generally inhibit aggressive responses in *C. floridanus* but instead specifically impacted workers' ability to discriminate NMs from nNMs and aggressively respond to the latter.

In order to further control for potentially confounding variables in response to these volatilization treatments, the activity of a single ant was recorded immediately following a 10-minute acclimation period. These trials consisted of a continuous 9-minute bioassay separated into three 3-minute segments. During the first segment, the ants were exposed to a continuous flow of

untreated air ('Acclimation'); for the second segment, the ants were exposed to a continuous flow of volatilized VUAA-class active or untreated air in the case of the blank control using the same parameters established for the volatilization aggression bioassay ('Treatment'); and during the third segment, the ants were again exposed to a continuous flow of untreated air ('Recovery'). A Y-junction connected to the compressed air tank alternated between the empty test tube during the Acclimation and Recovery phases and the treatment or blank tube during the Treatment phase. An examination of overall mobility parameters revealed no significant interaction effect when comparing control ants and ants treated with either an Orco agonist or antagonist before, during, or after exposure to each treatment (Figure III-3B-D, Table C-1).

Discussion

In ants and other eusocial insects, NM recognition depends on the ability to discriminate between self (NMs) and non-self (nNMs) (reviewed in (Sturgis and Gordon 2012)). While it is clear that these aggressive responses are mediated by the detection of chemical cues on the cuticle (Morel, Vandermeer, and Lavine 1988; Guerrieri et al. 2009; Leonhardt, Brandstaetter, and Kleineidam 2007; Neupert et al. 2018), the precise molecular mechanisms responsible for the detection and coding of that information within the olfactory system has remained ambiguous. Previous studies have demonstrated that the antennae are required for eliciting aggressive behaviors towards nNMs (Wang et al. 2016; Forel 1928). Therefore, we took a conservative approach to validate our aggression bioassay within the context of antennal ablations (Fig 1). Once established, this experimental paradigm was further adapted to accommodate the sustained volatile administration of VUAA-class Orco modulators to test the hypothesis that NM recognition in adult *C. floridanus* workers is solely dependent upon OR-based olfactory signaling as well as facilitate the characterization of odor coding in this process. Due to the broad developmental defects that result from the loss of Orco in other ant systems (Trible et al. 2017; Yan et al. 2017), these pharmacological tools provide a unique opportunity to acutely examine the role of OR-based signaling in a wild-type adult nervous system. At the same time, in light of the obligate colocalization of Orco together with tuning ORs in every insect ORN (Larsson et al. 2004; Jones et al. 2005; Taylor et al. 2012), exposure to Orco modulators is expected to have profound and widespread effects.

As previously observed in other contexts (Sharma et al. 2015), treatment with the VUANT1 antagonist effectively silences all Orco/OR complexes and prevents the generation of any interpretable signal (Figure III-2). In the case of the VUAA4 Orco agonist, activation of all Orco/OR complexes leads to either the activation of ORNs or a broad desensitization resulting in disrupted signaling (Figure III-2) that we postulate effectively generates an uninterpretable or "confused" coding signal. In either case, the lack of any odor signal or the presence of imprecise odor cues that are expected after treatment with an Orco antagonist or agonist, respectively, are both equally insufficient to elicit aggression between nNMs (Figure III-3).

The observation that an Orco antagonist decreases aggression between nNMs is broadly consistent with a simple U-present rejection model and supports the view that ants are not actively recognizing friends (Guerrieri et al. 2009; van Zweden and d'Ettorre 2010). However, the finding that an Orco agonist, which would be expected to generate a signal different from that of the endogenous template, would also decrease aggression between nNMs rather than increase aggression between NMs suggests that the simple presence of foreign yet imprecise cues are also insufficient to elicit aggression. These studies therefore support a model in which an unambiguous triggering stimulus must be precisely detected in order to evoke aggression. We propose that the recognition mechanism in *C. floridanus* occurs via a lock-and-key mechanism whereby the specific parameters of the foreign chemical label key, defined by the combinatorial presence and/or absence of salient odor cues, must be precisely decoded by an OR-mediated lock (Figure III-4). Under the assumption that a precise nNM label is compared to a neuronal template (of which multiple may exist), we conclude that ants may identify nNMs in two different ways which are not necessarily mutually exclusive: 1. As with previous models, unfamiliar nNM labels are compared to a familiar NM template and dissimilarity between these two leads to aggression (Neupert et al. 2018; Vander Meer and Morel 1998; Obin and Vandermeer 1989). However, given that neither VUANT1 nor VUAA4 elicited aggression, this dissimilarity must be constrained in some way with bounded thresholds wherein the label must be sufficiently different from the template but not so different as to be ambiguous; or 2. If unfamiliar nNM labels are compared to intruder templates that represent odor profiles which should be rejected from the colony and a certain level of precision between the label and template is required to elicit aggression then we would similarly expect both VUANT1 and VUAA4 to decrease aggression between nNMs.

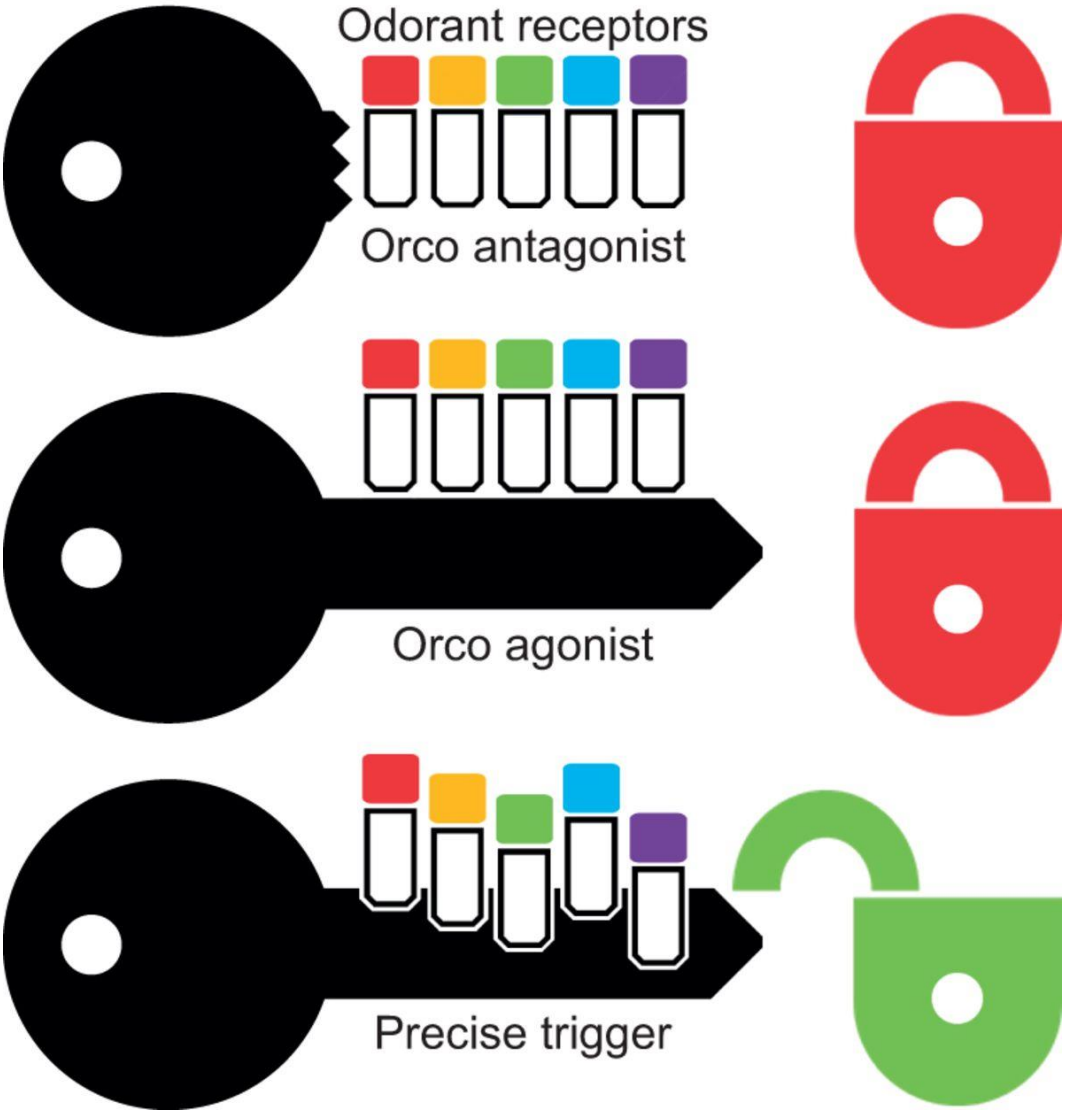

Figure III-4. Lock-and-key model of nNM recognition and aggression.
The triggering stimuli, represented by the teeth on a key, must be precisely detected by the OR tumblers in the lock. OR-dependent recognition of nNM cues leads to aggression against foes (green open lock); however, blocking OR-dependent recognition of NM/nNM cues does not lead to aggression, nor does the presence of an ambiguous chemical cue (closed red locks).

Furthermore, these data suggest that, when faced with some level of uncertainty, *C. floridanus* workers default towards acceptance rather than rejection. Over and above the benefits of conserving energy by avoiding potentially unnecessary aggression, for ants that spend the majority of their life cycles within colonies where they are more likely to encounter NMs than

nNMs, this strategy may also reduce acceptance errors and therefore increase overall colony fitness (Reeve 1989). It will be interesting to determine whether similar processes occur across worker behavioral task groups that may spend more time outside the nest (i.e. scouts and foragers) or whether different recognition methods have evolved across castes and/or species.

Our data definitively demonstrates that Orco/OR-mediated signaling is necessary for mediating aggression towards nNMs in *C. floridanus* and moreover excludes the sufficiency of other signaling pathways and sensory modalities in this context. These results are consistent with previous literature suggesting that aggression-mediated NM recognition may be more appropriately described as nNM recognition (Guerrieri et al. 2009; van Zweden and d'Ettorre 2010). While the roles of individual ant ORs or specific subsets of ORs in nNM recognition remain to be elucidated, the combinatorial interactions among specialized ORs (Slone et al. 2017; Pask et al. 2017), the plasticity of the neuronal templates (Neupert et al. 2018; Leonhardt, Brandstaetter, and Kleineidam 2007), the similarly diverse and plastic labels (Vander Meer, Saliwanchik, and Lavine 1989; Wagner et al. 1998; Kaib et al. 2000; Nascimento et al. 2013), and the observation that repeated stimulation with colony odors produced variable response patterns in the antennal lobe (Brandstaetter AS 2011) are likely to make those studies extremely challenging. Nevertheless, the demonstration that precise and unambiguous OR-based coding is necessary for ants to distinguish foe from friend represents a significant advance to link the longstanding interest in social insect behavior with more recent studies detailing the evolutionary complexity of the insect olfactory system (Hölldobler and Wilson 1990; Zhou et al. 2012; Zhou et al. 2015).

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Competing interests

The authors declare that they have no competing interests.

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Data availability

Data analysis and supporting information collected during this study are available in this published article (and its supplementary information files).

Authors' contributions

STF contributed to the design and conception of the work and was involved in collecting, analyzing, and interpreting the data as well as drafting and revising the manuscript. KYP and AR contributed to the conception and design of the experiments and assisted in data acquisition and analysis for the electrophysiology and aggression bioassay, respectively. IB assisted in data acquisition and analysis for the electrophysiology experiments. LJZ contributed to the design and conception of the work as well as interpreting the data and drafting and revising the manuscript. All authors read and approved the final manuscript.

Chapter IV. Olfactory Changes Associated with an Age Polyethism in the Eusocial Ant *Camponotus floridanus*[§]

Introduction

Ant colonies are complex adaptive systems that complete complicated tasks through the collective action of many workers (Bonabeau 1998). Behavioral patterns such as nursing offspring and foraging for food emerge as groups of ants detect and respond to local information such as chemical cues (Hölldobler and Wilson 1990). A single ant acting alone would therefore be unable to perform all the tasks necessary for colony survival. By the same token, ants reared in isolation have a significantly shorter lifespan compared to ants reared in the social environment of the colony (Koto et al. 2015). Rather, colony performance and survival depends on a dynamic and decentralized process of distributing work across all members of the colony (Gordon 1996). Understanding the rules that govern this process of task allocation are therefore important for understanding the emergent properties of coordinated social behavior in ants and other eusocial insect systems. Here, we characterize significant age and task associated shifts in olfactory sensitivity among two morphologically distinct worker castes in *Camponotus floridanus* colonies which may represent an important sensory mechanism by which tasks are allocated among workers.

In ant colonies, subtle changes to initial genomic or environmental conditions may give rise to individuals that vary dramatically in terms of their reproductive physiology, morphology, and lifespan (Hölldobler and Wilson 1990). Reproduction is often restricted to a limited number of individuals such as the queen(s) whose lifespan may be an order of magnitude greater than her offspring (Keller 1998; Keller and Genoud 1997; Hölldobler and Wilson 1990; Lucas et al. 2017). The remainder of tasks necessary for colony maintenance and survival are accomplished through the collective behavior of numerous, short-lived sterile female workers. The dynamic and decentralized process of distributing work across all members of a colony has been termed task allocation (Gordon 1996). Callow workers are typically confined to the safety of the nest where they tend to the brood before an age-associated transition to more dangerous tasks such as

[§] This chapter is data in preparation for submission with myself as first author, Isaac Bakis as middle author, and L.J. Zwiebel as senior author.

gathering food (Wilson 1976; Mikheyev and Linksvayer 2015; Tripet and Nonacs 2004). In this way, the transition from one task to another is accompanied not only by a dramatic change in environmental stimuli (i.e. chemical cues inside the nest compared to chemical cues outside the nest) but also a change in the relevant chemical releasers. For example, nurses differentiate queen-laid eggs from aberrant, worker-laid eggs within the colony through the detection of surface hydrocarbons on the eggs (Endler et al. 2004) whereas foragers follow trail pheromones to locate a food source outside the nest (Haak et al. 1996). Despite these radical shifts in ants' chemical environment over time, less is known about how variation in olfactory sensitivity among workers may regulate social behavior.

Importantly, task allocation is a dynamical process, and changes in colony need and environmental conditions influence the number and composition of workers engaged in a given task. For example, the experimental removal of subsets of workers from a colony leads to task switching (Tripet and Nonacs 2004). Group dynamics such as the number of workers available or the current proportion of the workforce engaged in a given task may also influence decision making (Wilson 1984; Gordon 1987). Importantly, the coordination of these social behaviors depends on dynamical interaction rates among workers which are intrinsically linked to the exchange of chemical cues. For example, the decision to leave the nest in search of food in harvester ants is determined by the rate at which successful foragers return to the nest (Greene and Gordon 2007). Information in this context is communicated through an olfactory-dependent process such that successful foragers are identified based on their cuticular hydrocarbon profile and the odor profile of the food (Greene, Pinter-Wollman, and Gordon 2013).

Different approaches have been taken to understand the regulation of task allocation. Thresholds models, for example, assume task performance depends on intrinsic differences between individuals resulting in thresholds that determine the probability of engaging in a task (Beshers, Robinson, and Mittenthal 1999). In support of these models, ambient humidity influences foraging activity in red harvester ant colonies as workers balance the need for water with the risk of desiccation (Gordon, Dektar, and Pinter-Wollman 2013). Alternatively, foraging-for-work models assume that workers will engage in tasks that they encounter when an insufficient number of workers are engaged in said task (Franks and Tofts 1994). However, empirical studies do not always support the model predictions. In *C. floridanus*, experimental nests comprised of only foragers randomly switch tasks consistent with a foraging-for-work model. In contrast, nests

comprised of only brood-tending workers exhibit an age-polyethism where the youngest workers tend to the brood and the oldest workers forage for food (Tripet and Nonacs 2004). Here, we use an empirical approach to characterize intrinsic physiological variation in the olfactory responses of major and minor *C. floridanus* workers.

Evidence for the importance of the chemical senses in mediating social behaviors comes from a long history of behavioral and chemical ecological studies which have been complimented by recent advances in targeted molecular approaches and gene editing techniques in ants. The detection of pheromones and other chemical signals occurs through three different chemoreceptor families. These notably include odorant receptors (ORs) (Gao and Chess 1999; Clyne et al. 1999; Vosshall et al. 1999; Fox et al. 2001), ionotropic receptors (IRs) (Benton et al. 2009; Abuin et al. 2011; Croset et al. 2010), and gustatory receptors (GRs) (Clyne, Warr, and Carlson 2000) which are expressed in olfactory sensory neurons (OSNs) in the antennae and other sensory appendages. Prescient genomic studies revealed that the OR family has rapidly evolved through a gene birth-and-death process that resulted in a large expansion of genes (Zhou et al. 2012; Zhou et al. 2015). Indeed, as of this writing, ants have the largest number of ORs among any insect species described to date. The GR family has also experienced a more modest level of gene gain and loss in certain ant species while the IR family less so. As a result, considerable effort has been dedicated towards functionally characterizing the ORs and understanding their role in social behavior.

In accordance with the hypothesis that a large expansion of chemoreceptors facilitated the evolution of eusocial insect colonies, it has been demonstrated that ORs are responsible for the detection of critical social cues, including general odorants and the all-important cuticular hydrocarbons (Slone et al. 2017; Pask et al. 2017). In *Harpegnathos saltator*, for example, at least three members of the 9-exon subclade of ORs (HsOr263, HsOr271, and HsOr259-L2) were capable of detecting 13,23 dimethylheptatriacontane, a putative queen pheromone. In addition, both 9-exon receptors and members of other OR subclades were responsive to cuticular extracts, unitary hydrocarbons, and general odorants such as ethyl acetate, 2,4,5-trimethylthiazole, and 2,3,5,6-tetramethylpyrazine. Knocking out the obligate OR co-receptor (Orco) gene which is required for the formation and function of the ligand-gated ion channel resulted in reduced colony cohesion and severe development alterations in the antennal lobe during development in two primitively eusocial ant species (Yan et al. 2017; Trible et al. 2017). Furthermore, targeted pharmacological modulation of Orco function in wildtype *C. floridanus* workers significantly

impaired aggression-mediated nestmate recognition (Ferguson et al. 2020). Taken together, these studies highlight the critical role ORs play in the detection of pheromones and other odorants that collectively mediate social behaviors in ants. However, less is known about the regulation of olfactory sensitivity with respect to task allocation and age polyethism.

Here, we extend these studies with a broad electrophysiological screen including more than 400 odorants across two morphological *C. floridanus* worker castes. Specifically, we test the hypothesis that changes in olfactory sensitivity are correlated with changes in age and worker task. We found that the majority of tasks are carried out by minor workers whereas majors may have a more niche role within the colony. Consistent with the higher level of task engagement, minor workers were also broadly and significantly more responsive to different odorants. However, CHCs and other hydrophobic compounds on the cuticle elicited strong responses from both majors and minors. This result emphasizes the important of CHC detection in ant colonies and suggests that majors may have a specialized role in nest defense. We also describe nuanced but meaningful olfactory response differences between approximately age-matched nurses and foragers. Specifically, nurses were significantly more responsive to 3-methylindole and were repelled by this odor at lower concentrations compared to foragers. Altogether, these findings suggest that changes in olfactory sensitivity may play an important role in regulating social behaviors in ant colonies.

Results

Morphological Castes Perform Different Tasks

In order to track the age and behavior of adult *C. floridanus* major and minor workers within the nest, callow workers were painted on the head, thorax, and gaster with a unique, painted color code. We considered the day the ants were painted to be day 0 because these ants presumably eclosed within the previous 24 hours. Sporadically, over a period of approximately one year, the behavior of painted workers engaged in various tasks was noted. These behaviors included nursing (carrying eggs or larvae), performing trophallaxis with nestmates, and foraging (drinking sugar water, eating crickets, or eating Bhatkar diet). As expected based on similar observations across many eusocial Hymenopteran including previous studies with *C. floridanus* (Wilson 1976; Mikheyev and Linksvayer 2015; Tripet and Nonacs 2004; Seeley 1982b; Kolmes and Sommeijer 1984; Jeanne, Williams, and Yandell 1992), minor workers exhibit an age-associated transition

from nursing (mean ± SEM = 20.83 ± 2.45 days old, N = 53) to foraging (51.69 ± 3.41 days old, N = 66) (Figure IV-1A). Ants performing trophallaxis with nestmates were of intermediate age (31.09 ± 4.65 days old, N = 22). The earliest time point that foraging was observed was day 6, and the majority of nursing behavior was observed before day 51. Workers in between these two time points were notably more plastic, and there was both nurses and foragers were observed at moderate to high levels between days 6-50. Based on these observations, for the purposes of this study, we organized our results into three bins: callows (days 1-5), plastic workers (days 6-50), and mature (days 51+).

In contrast to the relatively active minor workers which make up the majority of ants within the colony, majors rarely engaged in foraging and were almost never observed carrying brood (Figure IV-1B). It is important to note, however, that while we did not observe painted majors engaged in brood care, there was at least one instance where a non-painted major worker of unknown age was observed carrying a larvae. Nevertheless, such occurrences were quite rare. Majors engaged in trophallaxis (25.80 ± 5.83 days old, N = 5) and foraging (48.3 ± 13.51 days old, N = 10) were of similar age to their minor counterparts. Overall, the primary role of *C. floridanus* majors remains unknown, but these results suggest a more specialized function within the colony. For example, they may represent a soldier caste based on their enlarges mandibles, mobile larders due to their involvement in food consumption and trophallaxis, or as a "stand-by caste" if the population of minor workers is depleted (Wilson 1984).

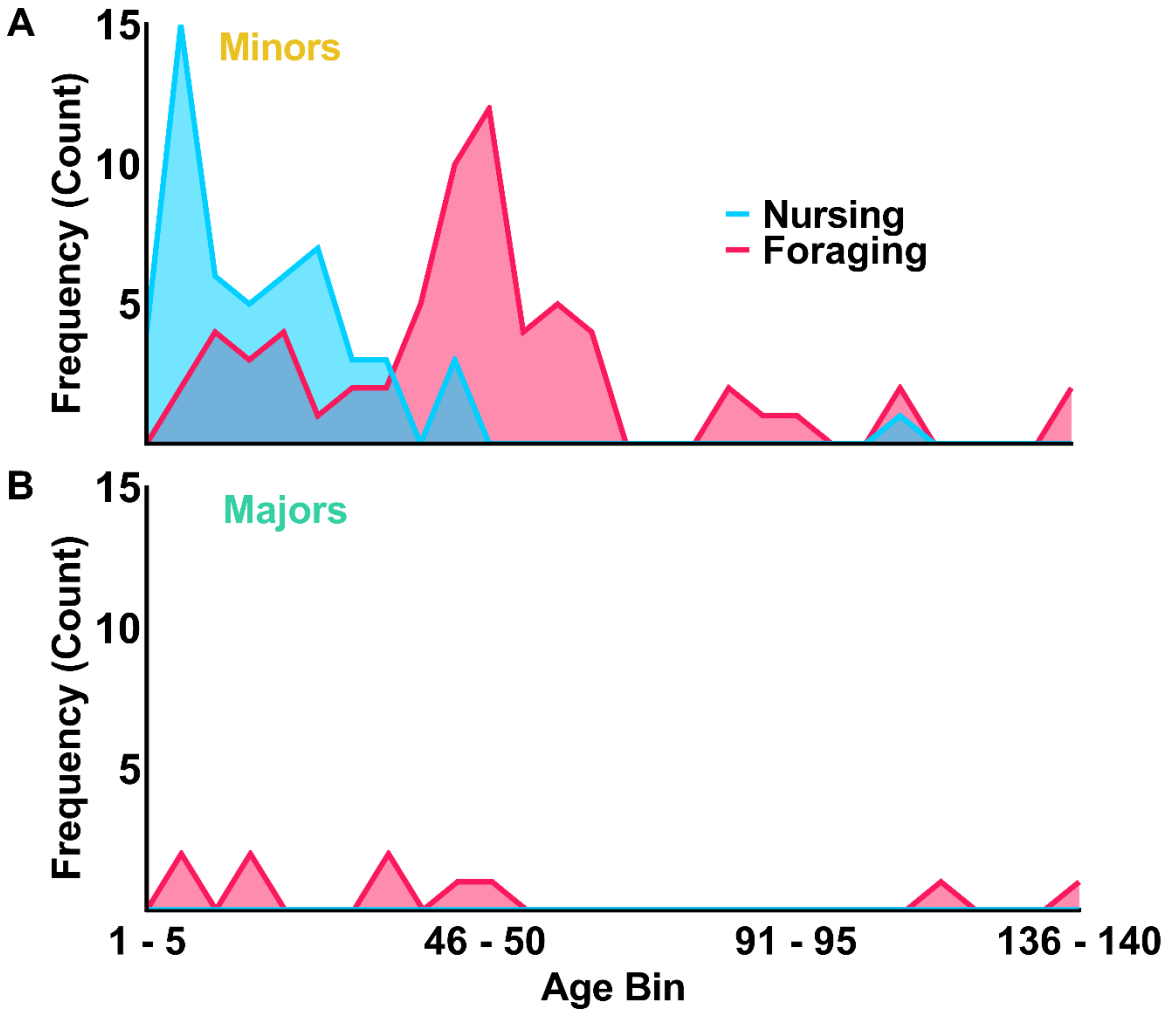

Figure IV-1. Caste-specific age polyethism among *C. floridanus* workers.
Minors and majors have a different repertoire of social behaviors. Minors exhibit an age-associated transition from nursing behavior (carrying eggs or larvae) (N = 53) to foraging (consuming crickets, Bhatkar diet, or sugar water) (N = 66) (A) whereas majors rarely nurse (N = 0) or forage (N = 10) (B).

Electrophysiology Setup and Establishing a Positive Control

In order to develop a consistent electroatennogram (EAG) protocol, we established a mounting setup that restricted worker movement to reduce noise and a positive control to ensure adequate contact prior to screening the odor library. Curiously, despite the impressive size of their antennae and the density of sensilla along the cuticle, EAG recordings in ants yield surprisingly low amplitude signals with a low signal-to-noise ratio compared to *Drosophila* which have

comparatively reduced antennae. By restricting the body, head, and mandibles of the ant in clay, we were able to obtain recordings with a satisfactory signal and minimal noise (Figure IV-2A-B).

In preliminary studies, we observed a robust dose-response to the amine 5,6,7,8-tetrahydroquinoline (TETQ) in both minors and majors (Figure IV-2C). From this data, we opted to use 10^{-1} M TETQ as a positive control before carrying out each EAG recording and set a threshold of at least 1.5x solvent (diethyl ether). If an adequate response to TETQ was not obtained, the experiment was simply not conducted. Throughout the studies reported in this manuscript, we recorded consistently high responses to TETQ with majors significantly more responsive relative to minors (Welch's t-test, df = 29.89, t = 2.22, P = 0.0341, N = 51 (minors), 23 (majors)) (Figure IV-2D). Overall, these results provide confidence in the consistency of recordings across different ants.

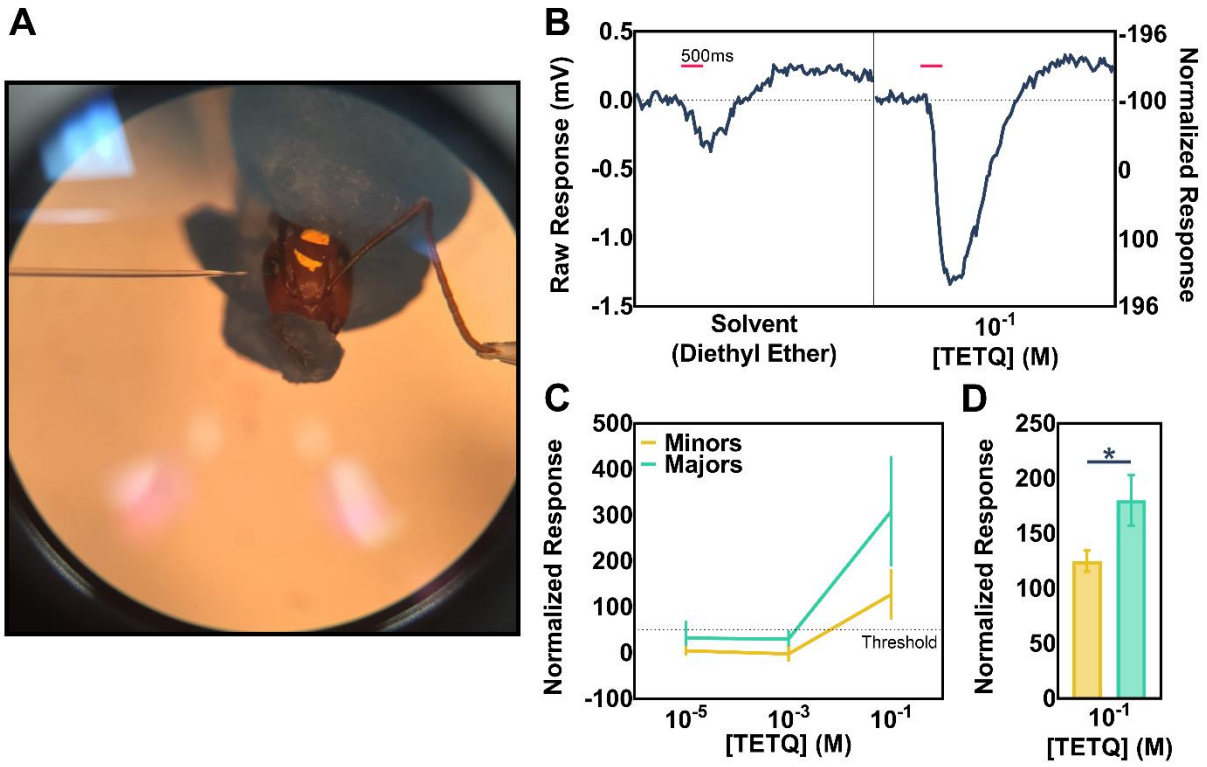

Figure IV-2. Electrophysiology setup and TETQ positive control.
An overview of the EAG mount and positive control. A representative EAG mount demonstrating the use of clay and strategic positioning to secure the head, mandibles, and antennae of a minor worker (A). Representative EAG traces in response to solvent (diethyl ether) (left) and TETQ (right) (B). Preliminary results demonstrating that both minors and majors (N = 5) displayed dose-response to serial dilutions of TETQ with a dotted line indicating the minimum

threshold for our pre-experiment positive control (C). Across all EAGs reported in this manuscript, both minors and majors displayed above-threshold responses to 10^{-1} M TETQ, and majors were significantly more responsive to this compound relative to minors (Welch's t-test, df = 29.89, t = 2.22, P = 0.0341, N = 51 (minors), 23 (majors)) (D).

Low Base Responsiveness of the Antennae in Majors and Callow Minors

After establishing electrophysiological contact, we next sought to screen a large panel of odorants. In total, we screened 406 odorants across 10 different chemical classes which were organized into 36 different blends. Each blend contained between 9 and 17 odors. Based on the cumulative raw responses acquired across all 36 blends, we found that callow minor workers were significantly less responsive to odorants relative to plastic and mature minors (Two-Way ANOVA with Tukey's correction for multiple comparisons, P < 0.0001, N = 36) (Figure IV-3A). Indeed, the raw response to every blend tested was lower in callows compared to both plastic and mature minors. These results are broadly consistent with experiments conducted using two different honeybee species that demonstrated low abundance of certain olfactory genes in callow workers (Zhao et al. 2016; Nie et al. 2018). Majors had relatively lower responsiveness overall (Figure IV-3A) and across all age ranges tested were most similar to callow minors (Figure IV-3B). Curiously, normalized responses to the positive TETQ control in majors were significantly higher compared to minors (column (minor v. major) factor Two-Way ANOVA, df = 1, F = 6.29, P = 0.0145, N = 51 (minors), N = 23 (majors)) (Figure IV-3C). Taken together, these results suggest that the base responsiveness of the antennae is higher in older minor workers compared to callow minors and majors irrespective of the odor compounds being tested.

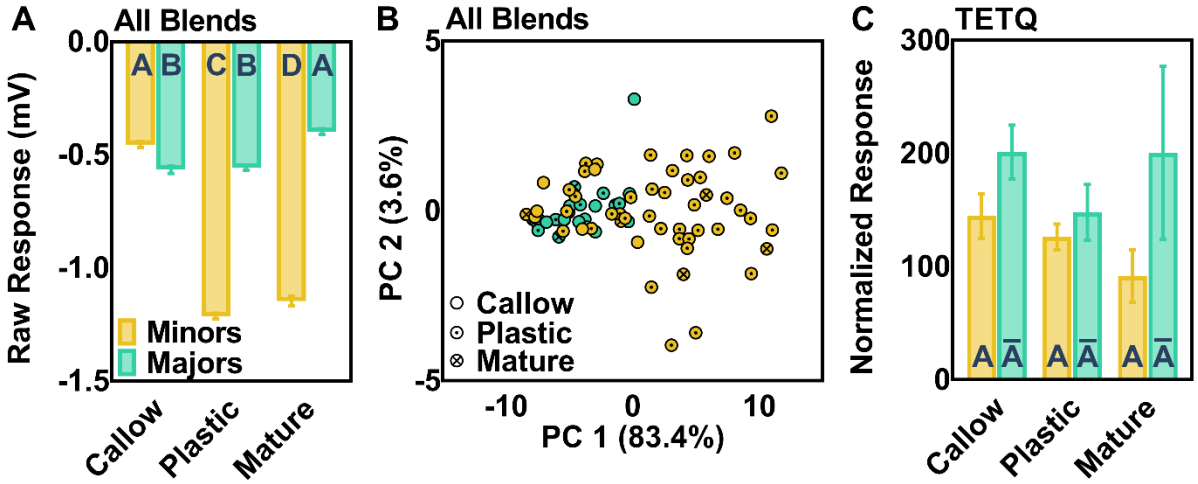

Figure IV-3. Low baseline sensitivity in majors and callow minors.
Majors across all age ranges tested have similar, low baseline olfactory sensitivity compared to callow minors despite significantly higher normalized responses to the positive TETQ control. A comparison of the average olfactory response to each of the 36 odor blends (Two-Way ANOVA with Tukey's correction for multiple comparisons, $P < 0.0001$, $N = 36$) (A). PCA based on the olfactory responses to all 36 odor blends across callows (open circles), plastic workers (circle with dot), and mature workers (circle with 'x') (B). A comparison of the normalized responses to the positive control TETQ (the column (minor v. major) factor was significant and denoted by the A v. A bar, but the posthoc comparisons were not significant, Two-Way ANOVA, $df = 1$, $F = 6.29$, $P = 0.0145$, $N = 51$ (minors), $N = 23$ (majors)) (C).

Majors are Less Responsive to Odor Blends Compared to Minors

After normalizing the EAG responses to solvent (ND96), we found that majors were still broadly less sensitive to the various odor blends tested. Only blends 4, 6, 7, and 28 were higher in majors and only by a small margin. This striking difference in olfactory sensitivity may reflect the reduced social behavior repertoire we observed in majors (Figure IV-1B) and that has been reported in the major workers of other ant species (Wilson 1984). It is curious, however, that the diminished olfactory responses among majors are not simply null responses (= 0) comparable to the solvent. Rather, there are many large inhibitory responses, especially among the carboxylic acids (blends 15-18) (Figure IV-4A), and it is possible that these also represent biologically meaningful signals in their own right.

Principle component analysis suggested that a modest degree of variance (approximately 30% across PC1 and PC2) could be explained by the various chemical classes tested (Figure IV-4B-C). For both minors (along PC1) and majors (along PC2), alcohols and aldehydes clustered together and were separate from esters, ketones, lactones, and sulfides. Amines, carboxylic acids, and esters also tend to cluster in-between the various other chemical classes. Altogether, these trends suggest that, even though majors are less responsive to odors overall, the patterns of relative excitation and inhibition are broadly similar between minors and majors.

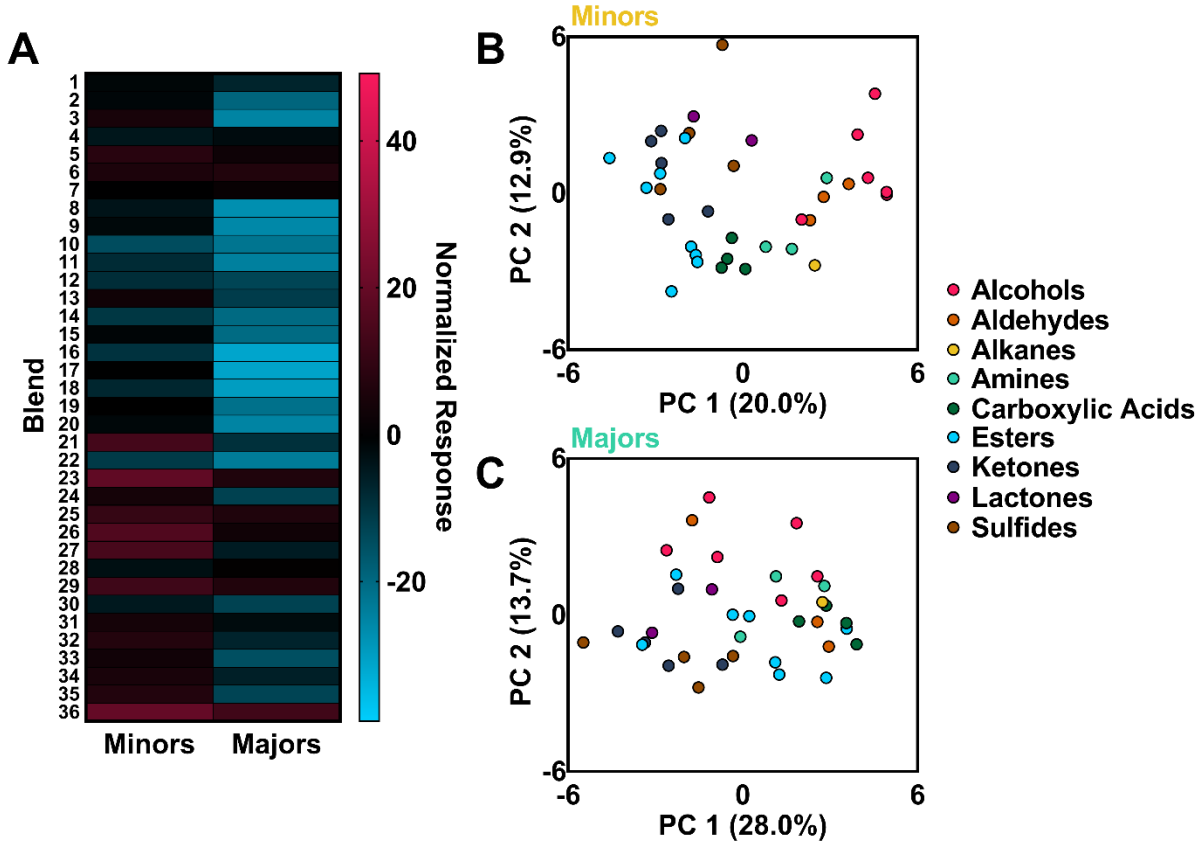

Figure IV-4. Majors are less responsive to odor blends compared to minors.
Majors are broadly less sensitive to odor blends compared to minors, and there are different trends in the responses to certain chemical classes between minors and majors. A heatmap of the average responses of aging minors and majors (A). PCA analysis of the various chemical classes analyzed along with the percentage of variance explained by PC1 and PC2 (B-C).

Age-Associated Shifts in Olfactory Sensitivity

In order to examine age-associated changes in olfactory sensitivity, we binned worker responses from callows (days 1-5, no foraging behavior observed), plastic workers (days 6-50, both nursing and foraging behavior observed), and mature workers (days 51+, primarily foraging behavior observed). We found a number of age-associated changes in responsiveness in both minors and majors (Figure IV-5A). For minors, there was a significant increase in responsiveness to alcohol blend 1 (One-Way ANOVA with Tukey's correction for multiple comparisons, P = 0.0446, N = 26 (plastic), N = 5 (mature)) (Figure IV-5B). The highest response to any blend tested was observed in callow workers elicited by the ketones and indoles in blend 26, and this blend showed a significant age-associated decrease in sensitivity (One-Way ANOVA with Tukey's

correction for multiple comparisons, P = 0.0099, N = 6 (callow), N = 5 (mature)). Majors displayed similar and significant age-associated shifts in sensitivity such as an increase in responsiveness to the amines in blend 13 (One-Way ANOVA with Tukey's correction for multiple comparisons, P = 0.0110, N = 9 (plastic), N = 6 (mature)) and a decrease in responsiveness to the lactones in blend 31 (One-Way ANOVA with Tukey's correction for multiple comparisons, P = 0.0127 (callow v. plastic), P = 0.0184 (callow v. mature), N = 6 (callow), N = 9 (plastic), N = 6 (mature)). However, the magnitude of these responses was less pronounced than that of minors.

We then focused our analysis on the unitary compounds in blend 26. We selected this blend because it elicited the highest overall response in callow minors and the lowest overall response in mature minors, suggesting that the compounds in this blend may be involved in nursing as this is often performed by younger workers and less so by older workers (Figure IV-1A). Contrary to our expectations, however, the responses to the unitary compounds were not significantly different between callows and mature minors, and several compounds elicited slightly higher responses in mature minors (Figure IV-5C). These results may reflect nuanced olfactory response differences that arise in response to unitary compounds, which are less likely to be encountered in a biologically relevant setting, compared to odor blends, which are significantly more complex but more commonly encountered in nature.

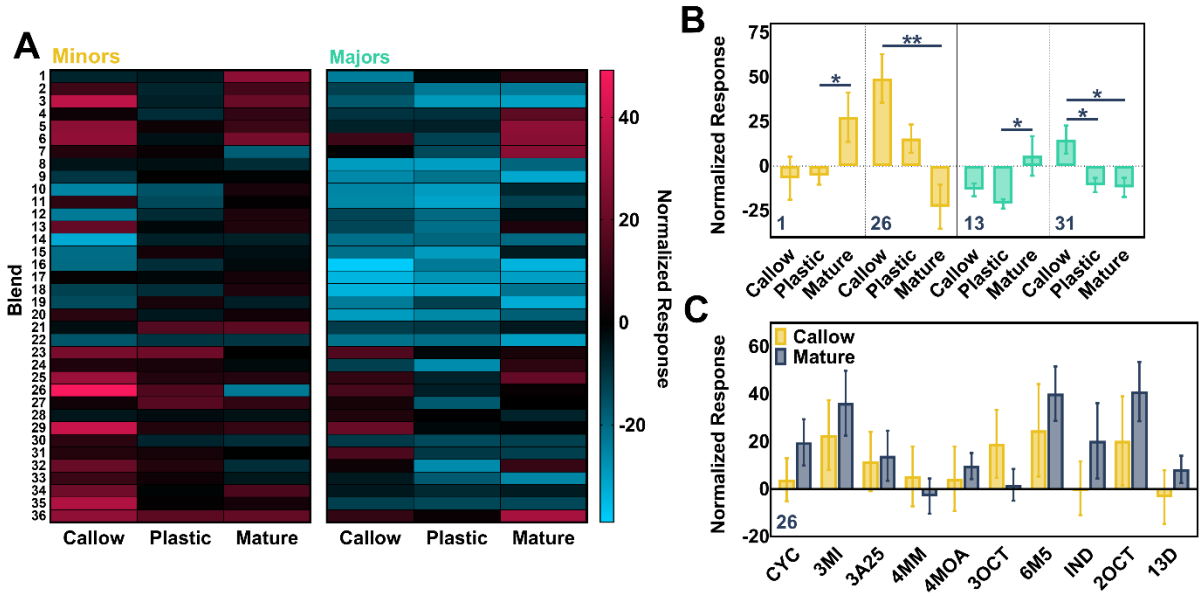

Figure IV-5. Age-associated shifts in olfactory sensitivity in minors and majors.
There are both age- and caste-associated shifts in olfactory sensitivity. A heatmap of the average responses of minors and majors binned into callow (day 1-5), plastic (day 6-50), and mature (day 51+) groups (A). Select comparisons of

compounds that show significant age-associated differences in responsiveness in minors (yellow) and majors (green). EAG responses to the unitary compounds in blend 26 in callow (yellow) and mature (blue) minor workers.

Task Associated Shifts in Olfactory Sensitivity

While there is a tight association between age and behavior in *C. floridanus* (Figure IV-1) and other eusocial insects (Wilson 1976; Mikheyev and Linksvayer 2015; Tripet and Nonacs 2004), it is not possible to determine whether the differences we observed in olfactory sensitivity across callow, plastic, and mature workers was due to age- or task-associated changes. To address this problem, we collected approximately age-matched minor nurses (mean ± SEM = 34.71 ± 3.00 days) and minor foragers (mean ± SEM = 27.71 ± 4.84 days) from the plastic age range (days 6-50) and screened these workers using the same EAG protocol as above. Overall responses between nurses and foragers were similar (Figure IV-6A) suggesting that a broad degree of olfactory sensitivity may facilitate task switching among *C. floridanus* workers. There were, however, more nuanced response differences to unitary odorants. In keeping with our focused examination of the unitary compounds in blend 26, we found that nurses were significantly more responsive to 3-methylindole compared to foragers (Two-Way ANOVA with Tukey's correction for multiple comparisons, $P < 0.0141$, $N = 7$) (Figure IV-6B), suggesting this compound may be responsible for task-associated differences in behavior.

To functionally characterize the valence of 3-methylindole, we first examine the response of whole colonies to Whatman paper strips containing either solvent (DMSO) or serial dilutions of 3-the compound. The ants as a collective exhibited a dose-dependent avoidance behavior in response to increasing concentrations of 3-methylindole, and there were significantly less ants walking on the Whatman paper containing 10^{-1} M 3-methylindole compared to the solvent control (One-Way ANOVA with Tukey's correction for multiple comparisons, $P = 0.0038$, $N = 7$). Next, we examined the valence of individual minor worker nurses and foragers. Throughout this assay, we found that foragers had a tendency to walk a greater distance compared to nurses, although this was not statistically significant (Figure IV-6C).

Nurses often moved away from the Whatman paper containing 3-methylindole at every concentration tested whereas foragers appears to have a dose-dependent avoidance reponse (Figure IV-6D). As such, nurses were located significantly father away from the treatment zone compared to foragers at the lowest concentration tested (10^{-5} M) (Two-Way ANOVA with Tukey's

correction for multiple comparisons, $P < 0.0001$, $N = 8$). Furthermore, foragers spent significantly more time standing directly on the Whatman paper at the 10^{-5} M concentration compared to nurses (Two-Way ANOVA with Tukey's correction for multiple comparisons, $P = 0.0154$, $N = 8$).

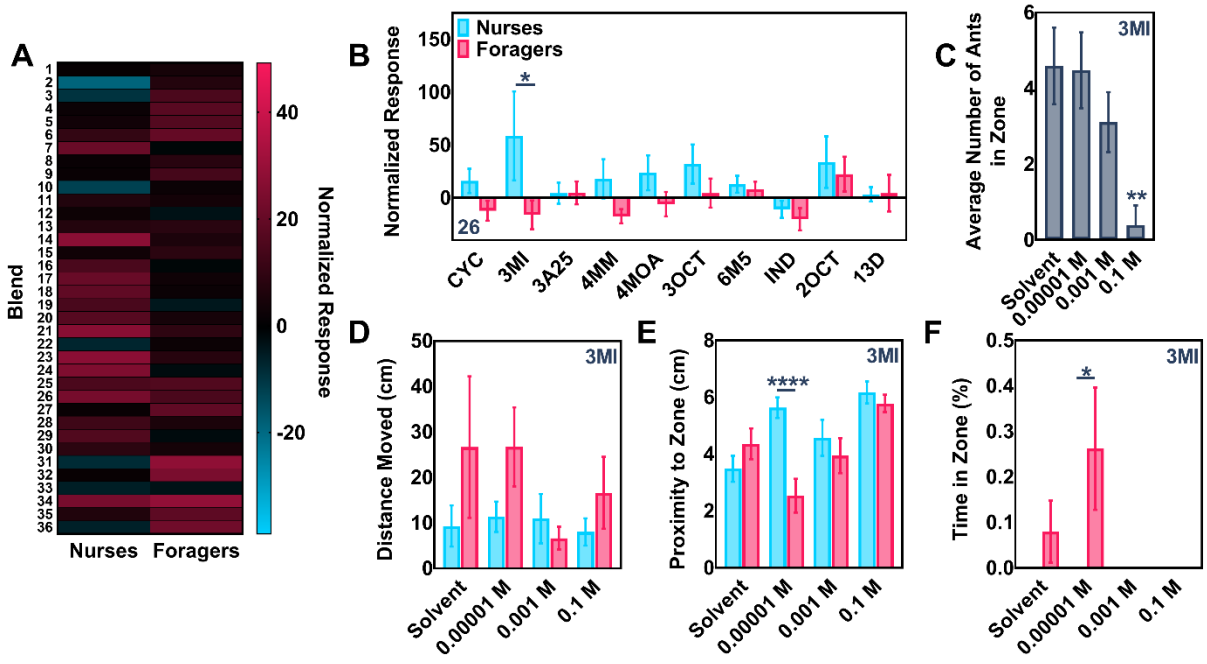

Figure IV-6. Task-associated shifts in olfactory sensitivity in nurses and foragers.
Subtle but meaningful olfactory differences between approximately age-matched nurses and foragers. A heatmap of the average responses of minor nurses and foragers (A). EAG responses to the unitary compounds in blend 26 in nurses (blue) and foragers (red) (B). Colony-level valence in response to various concentrations of 3-methylindole (C). Valence of nurses (blue) and foragers (red) in response to various concentrations of 3-methylindole (D-F).

Cuticle Extract Elicits High Responsiveness from both Minors and Majors

Finally, to characterize olfactory responses to CHCs and other odorants commonly encountered on the cuticles of nestmates and non-nestmates, we screened a hydrophobic soak equivalent to half of an ant obtained from nestmate and non-nestmate minors and majors. While not directly comparable, soak from all combinations of colony and caste elicited extremely high responses from both minors and majors (Figure IV-7). While majors were still slightly less responsive compared to minors, the overall responses are similar, and the high responsiveness in majors directly contrasts the broad inhibitory responses observed in response to the different odor blends. It is therefore tempting to speculate that majors may represent a soldier caste within the colony

with a specialized olfactory sense dedicated primarily to the detection of CHCs that communicate colony membership.

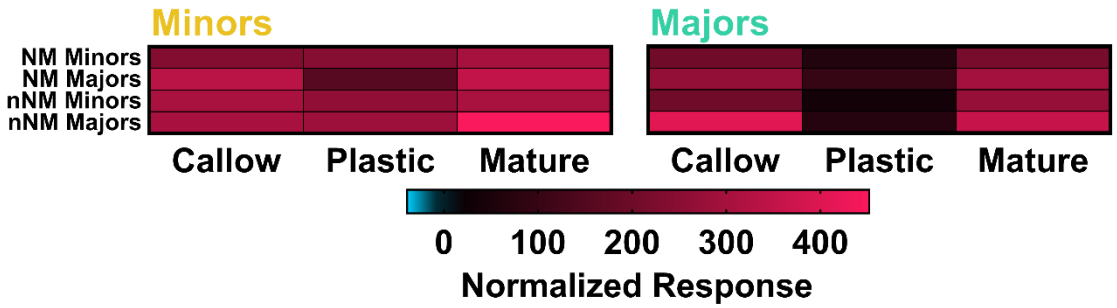

Figure IV-7. High olfactory sensitivity to cuticle extract in both minors and majors.
Both minors and majors exhibit high sensitivity to cuticle extracts from nestmate (NM) and non-nestmate (nNM) minors and majors.

Discussion

Collective social behaviors in ant colonies emerge as workers are allocated to different tasks. Callow workers often reside in the nest, in close proximity to the queen, where they tend to the brood. These nurses provide food to the larvae and shift the brood pile in response to changes in ambient temperature (Porter and Tschinkel 1993). Older workers take on more dangerous roles such as foraging for food outside the nest. However, age is likely only a correlate of task because changes in colony need, for example the removal of certain workers (Tripet and Nonacs 2004; Crall et al. 2018), may also influence worker behavior. Importantly, these behavioral transitions are accompanied by a dramatic shift in the chemical and ecological environment of the workers. The chemical environment in the nest is much different than that outside the nest, and there are different chemical cues that release nursing and foraging behavior (Endler et al. 2004; Haak et al. 1996). Here, we report a comprehensive electrophysiological screen of minor and major *C. floridanus* workers that examines age, caste, and task-associated variation in olfactory sensitivity.

As one would predict from the abundance of literature on age polyethism in eusocial insects, *C. floridanus* minor workers primarily tend to the brood as callow workers and begin foraging as they mature (Wilson 1976; Mikheyev and Linksvayer 2015; Tripet and Nonacs 2004). However, there were periods of relatively high plasticity when minors of a similar age

may engage in either nursing or foraging behavior. Majors, on the hand, rarely engaged in either nursing or foraging. This reduced repertoire of social behaviors among majors is similar to that reported in many Pheidole species (Wilson 1984). Consistent with these observations, callow minors had significantly low sensitivity to all odor blends tested but displayed greater sensitivity as they aged. Moreover, majors remained less responsive over the same age ranges. However, both minors and majors displayed significantly high responses to chemical blends such as CHCs and other hydrophobic odorants found in cuticular extracts. While both minor and major workers aggress non-nestmates, this restricted olfactory response in major workers suggests a role in nest defense as a soldier caste.

Overall, approximately age-matched nurses and foragers had relatively similar olfactory response profiles. Based on this result, we speculate that flexible task allocation may be facilitated when workers maintain broad olfactory sensitivity. However, the precise biological relevance of each compound in this screen are less clear, and it's possible that only a subset are involved in task-specific social behaviors. Furthermore, we observed significant, if nuanced, differences between nurses and foragers. Namely, nurses were significantly more responsive to 3-methylindole relative to foragers. This indole, also known as skatole, is found in the feces of certain animals and gives army ants a characteristic fecal odor (Brown, Watkins, and Eldridge 1979). For orchid bees (Schiestl and Roubik 2003; Leal et al. 2008) and gravid mosquitoes, this compound is an attractant. In *C. floridanus*, however, we found that at the colony-level, high concentrations of this compound result in a repellent behavior. In addition, nurses, who are significantly more sensitive to this odorant, were repelled at a lower concentration relative to foragers. That differences in responsiveness to a given odorant among workers of a similar age may be responsible for phenotypic behavioral differences suggests that variation in olfactory sensitivity may play an important role in task allocation in ant colonies.

Conclusions

This report represents, to our knowledge, the largest electrophysiological screen conducted in an ant species. Here, we assayed the olfactory responses of two morphological castes to a large odor library (>400 odorants) within the context of age polyethism. From this comprehensive examination, we found significant differences in the olfactory responses between minors and majors that correspond to the apparent size of their social behavior repertoire. We

also discovered subtle yet significant differences in responsiveness to 3-methylindole and further demonstrated that this compound has significantly different valence between age-matched nurses and foragers. Altogether, these data support the hypothesis that changes in olfactory sensitivity may play an important role in the regulation of task allocation in eusocial ants.

Methods

Animal husbandry

Eight laboratory-reared colonies of *Camponotus floridanus* (Buckley 1866) originating from field collections by Dr J. Liebig (Arizona State University) from the Sugarloaf Key (D601), and Dr S. Berger (University of Pennsylvania) from the Fiesta Key (C6, K17, K19, K28, K31, K34 and K39) in South Florida, USA were separately maintained at 25°C and an ambient humidity of approximately 70%. Colonies were stored in an incubation chamber with a 12 h light:12 h dark photoperiod. Each colony was provided with Bhatkar diet, crickets, 10% sucrose solution, and distilled water 3 times per week. Adult minor and major workers were used for all experiments.

Paint marking, behavioral monitoring, and collections

Callow workers were identified based on their soft, light-colored cuticle, low mobility, and proximity to the brood pile. Ants with these characteristics were likely to be less than 24 hours post-eclosion at the time of collection. These callow workers were briefly anesthetized with CO_2 and Sharpie oil-based paint pens were used to mark the head, thorax, and gaster with a unique color code. Prior to making behavioral observations, colonies were removed from the incubation chamber and allowed to acclimate after handling for at least five minutes before behavioral observations were made. If the colony was disturbed, for example when removing trash or replacing food, no observations were made that day. The behavior of age-known ants engaged in pre-specified behaviors (carrying eggs, carrying larvae, performing trophallaxis, eating crickets, eating Bhatkar, and drinking sugar water) that could be identified based on their paint code was then recorded as observed. To collect ants for subsequent downstream electroantennography and behavioral bioassay analyses, individual paint marked ants were randomly sampled from among the different colonies tested. When specifically selecting for

nurses and foragers, ants actively engaged in carrying eggs or larvae or consuming crickets, Bhatkar diet, or sugar water were selected, respectively.

Electroantennography

Electroantennograms (EAGs) were conducted using an IDAC-232 controller (Ockenfels Syntech GmbH, Buchenbach, Germany) and data was initially collected and stored on EAG2000 software (Ockenfels Syntech GmbH). Odorants were delivered using a Syntech CS-05 stimulus flow controller (flow rate of 1.5 cm^3 s^{-1}; Ockenfels Syntech GmbH). Minors were placed in a 20 µl disposable pipette tip that was modified such that the tip opening was sufficiently wide to allow the unimpeded exposure of the head and antennae. Majors were placed in modified 200 µl pipette tips to accommodate their wider head. To prevent unwanted movement from the ant that might otherwise interfere with the quality of the recording, the head and mandibles of the ant were restricted with wax in addition to the right antennae. Borosilicate glass capillaries (FIL, o.d. 1.0 mm, World Precision Instruments, Inc.) were prepared using a P-2000 laser micro-pipette puller (Sutter Instruments). Both the reference electrode and the recording electrode were backfilled with 10^{-1} mol l^{-1} KCl and 0.05% PVP buffer and placed over tungsten electrodes. Due to the armor-like exoskeleton of the ant, a 30-gauge needle was required to puncture the right eye prior to inserting the reference electrode. The recording electrode was placed over the distal tip of the left antenna. A representative example of this preparation can be seen in Figure IV-2A.

The TETQ positive control was diluted in diethyl ether at a concentration of 10^{-1} M. Odor cartridge were prepared with 10 µl of solution. Prior to all electrophysiological recordings, the ant was first exposed to diethyl ether, then TETQ, and then another exposure to diethyl ether. Normalization of the TETQ response was then accomplished through linear interpolation vis-à-vis EAG2000. If the response was greater than 1.5x solvent, then the experiment would commence. Otherwise, the experimental recording was not conducted. Odor blends were diluted in ND96 buffer, and individual odorants were diluted in DMSO. Each odorant was diluted to a concentration of 10^{-3} M. Odor cartridge were again filled with 10 µl of solution. Recordings were conducted in the following order: ND96, odor blends 1-18, ND96, odor blends 19-36, ND96. In the case of individual odorants, the recordings were conducted as follows: DMSO, individual odorants, DMSO. Cuticular extracts were obtained by soaking 10 nestmates, either minors or majors, in 2 ml of hexane for 30 minutes. This cuticle soak was then decanted, and the hexane

was evaporated using compressed nitrogen. The remaining contents of the extraction were then resuspended in 400 µl of hexane. Odor cartridges were then filled with 20 µl of hexane or hexane plus extract, equivalent to cuticle soak obtained from half of an ant. When testing odor responses, a handheld butane torch (BernzOmatic, Worthington Industries, Columbus, OH, USA) was used to volatilize the compounds by heating the odor cartridge for 1.5 s. Odors were introduced in the following order: hexane, cuticle extract, hexane. In this way, responses could be normalized to solvent responses recorded across the duration of the trial to account for antennae degradation over time throughout the assay. Both the raw data and normalized responses were used for subsequent data analysis.

Behavioral Bioassay - Colony-Level Responses to 3-methylindole

To assay colony-level responses to 3-methylindole, small pieces of Whatman paper (2.5 cm in diameter) were soaked in 50 µl of solvent (DMSO) or serial dilutions of 3-methylindole (10^{-5}, 10^{-3}, or 10^{-1} M). These Whatman paper were then randomly placed in the colony, and behavior was recorded for 10 minutes using a digital high-definition camera (Panasonic® HC-V750). Videos were then analyzed posthoc and the number of ants on each piece of paper was recorded every 30 seconds and averaged across the duration of the trial.

Behavioral Bioassay - Individual Responses to 3-methylindole

In order to assay individual responses to 3-methylindole, nurses (carrying eggs or larvae) and foragers (consuming crickets, Bhatkar diet, or sugar water) were collected and placed into modified Petri dish arenas (150 mm in diameter). The lid of these arenas had a single, small hole (1 cm in diameter) near the edge. A small square of mesh secured with double sided sticky tape was placed over the top of this hole to allow ventilation of the arena. Prior to the start of the bioassay, a single ant was placed underneath a small lid (35 mm in diameter) in the Petri dish arena and allowed to acclimate for 10 minutes. After this acclimation period, a small piece of Whatman paper (2.5 cm in diameter) soaked in either solvent (DMSO) or a serial dilution of 3-methylindole (10^{-5}, 10^{-3}, or 10^{-1} M) was introduced into the arena underneath the ventilation hole. The lid was then removed and the ant allowed to wander freely in the arena. The location of the ant was then digitally recorded for 10 minutes, and these videos were analyzed using an automated tracking software package (EthoVision® XT v8.5, Noldus Information Technology,

Wageningen, The Netherlands) to calculate total distance traveled (cm), proximity to the Whatman paper zone (cm), and the proportion of time spent standing directly on the Whatman paper zone.

Statistical Data Analysis

Statistical data analysis including the creation of figures was performed using GraphPad Prism v7.03 (GraphPad Software, Inc.). A Welch's t test was used to compare the overall TETQ responses between minors and majors (Figure IV-2D). A Two-Way ANOVA with Tukey's correction for multiple comparisons was used to analyze the raw responses of the various odor blends (Figure IV-3A), the normalized responses to TETQ between minors and majors (Figure IV-3C), the unitary odorant responses from Blend 26 (Figure IV-5C and Figure IV-6B), and the movement and location data derived from EthoVision for individual responses to 3-methylindole (Figure IV-6D-F). One-way ANOVAs with Tukey's correction for multiple comparisons were used to analyze the responses to select odor blends (Figure IV-5B) and the colony-level responses to 3-methylindole (Figure IV-6C). PCA was performed using ClustVis (Metsalu and Vilo 2015). The data for PC1 and PC2 were then imported into GraphPad for figure creation.

Chapter V. Advances in the Study of Olfaction in Eusocial Ants[**]

Abstract

Over the past decade, beginning with the sequencing of the first ant genomes, there have been major advances in the field of olfactory myrmecology. With the discovery of a significant expansion of chemoreceptors in the odorant receptor gene family, considerable scientific effort has been directed towards understanding the olfactory basis of complex social behaviors in ant colonies. Here, we review recent pivotal studies including investigations of odor coding in the antennae and central brain, the ontogeny of the antennal lobe glomeruli, and progress in the application of gene editing and other molecular techniques that notably distinguish the complex olfactory system of ants from other well-studied insect model systems including the fruit fly. In doing so, we hope to not only highlight the extraordinary scientific developments but also draw attention to critical knowledge gaps that will serve as a compass for future research endeavors.

Introduction

A Remarkable Olfactory Sense

From an evolutionarily perspective, ants are an extraordinarily successful insect taxa that are globally pervasive and comprise more than a quadrillion individuals to effectively dominate a significant proportion of the terrestrial biomass (Hölldobler and Wilson 1990). It is likely this success is largely due to the complex eusocial structures that drive collective behavior among individuals throughout a colony. In the absence of centralized control, sterile female workers tend to the queen's offspring, construct nests, defend and police the colony, and search for food. Beyond these fundamental tasks which are commonly observed across eusocial insect taxa, the social life of certain ant species may be quite extraordinary. Attine ants rely on the collection of leaves which they use as a substrate to maintain elaborate fungal gardens (Weber 1972). Army ants create living nests with their bodies known as bivouacs where they shelter the queen and store food and brood among the interior chambers (Kronauer 2020). When selecting a new nest site, rock ants engage in a democratic decision making process that relies on quorum sensing (Pratt et al. 2002). To accomplish these impressive feats, ants largely rely on sophisticated chemical communication

[**] This chapter is in preparation for submission to a special edition of *Insects* with myself as first author. Isaac Bakis will be a co-author. L.J. Zwiebel will be senior author.

systems that provide an extraordinary degree of discrimination and sensitivity (Ferguson, Anand, and Zwiebel 2020).

Ants communicate with one another by exchanging an array of chemical messages. Many of these messages are detected via olfactory signal transduction pathways largely localized to the antennae (Ferguson, Anand, and Zwiebel 2020). Complex blends of cuticular hydrocarbons (CHCs) are an especially important class of semiochemicals that convey a broad range of social information including colony membership, fertility, and task group (Sprenger and Menzel 2020). In the course of a brief antennation, an ant can identify a foraging nestmate or an intruding non-nestmate based on their respective CHC profiles (Greene, Pinter-Wollman, and Gordon 2013; Morel, Vandermeer, and Lavine 1988). CHCs are produced by oenocytes associated with the fat body (Arrese and Soulages 2010; Roma, Bueno, and Camargo-Mathias 2010; Fan et al. 2003), and it is believed that the postpharyngeal gland plays a central role in storing and distributing the hydrocarbons involved in colony identity (Soroker and Hefetz 2000). Indeed, there is considerable qualitative and quantitative similarities between the contents of the PPG and the CHC profile (Bagnères and Morgan 1991). Taken together, these studies highlight only a small fraction of the complex pheromone biochemistry responsible for the organization and coordination of ant societies.

Over and above the role of CHCs, ants have been described as "walking chemical factories" because they rely on a large array of exocrine glands that collectively produce the semiochemical releasers for many complex social behaviors (Hölldobler and Wilson 1990). For example, in *Formica argentea*, undecane is produced in high concentrations in the Dufour's gland where it is likely to act as an alarm pheromone component (Lenz, Krasnec, and Breed 2013). In addition to its role in predation and defense, the poison gland of Formicidae produces formic acid which may act synergistically with other compounds that elicit alarm responses in the Dufour's gland (Löfqvist 1976; Fujiwara-Tsujii et al. 2006). Even more notorious is fire ant venom which is comprised of hydrophobic dialkylpiperidines known as solenopsins used for predation and defense (Fox 2014). Beyond these select examples, there is tremendous diversity of exocrine gland form, function and output among ants, including a range of evolutionarily-derived glands that may elicit behaviors that are unique to a given genera (Hölldobler and Wilson 1990). Moreover, even closely related species may have strikingly different exocrine gland composition. This is illustrated in studies that examined the phylogenetic relationship of *Camponotus floridanus* and *Camponotus*

atriceps which was contested for a time with some scholars suggesting that the two species were synonymous (Hashmi 1973). However, the ratio of compounds in the Dufour's gland was observed to be notably different, with certain compounds, such as 2-methyldecane and heneicosane, being present in only one species or the other, respectively (Haak et al. 1996). The distinct phylogeny is also consistent with studies demonstrating that trail following behaviors are evoked by distinct hindgut components found in each species. In this regard. *C. floridanus* is sensitive to nerolic acid while *C. atriceps* relies upon 3,5-dimethyl-6-(1'-methylpropyl)-tetrahydropyran-2-one.

Aim and Scope of this Review

The availability of the first ant genome sequences (Bonasio et al. 2010) revealed that ants have greatly expanded gene families of specialized odorant receptors (ORs) than any insect species described to date (Zhou et al. 2012; Zhou et al. 2015) have fostered several studies to bridge the gap between ant chemical ecology and the underlying molecular machinery responsible for these complex eusocial interactions. While there is a considerable body of literature on the complex life-cycles and biology of ants, this review will focus on what we consider to be the major advances in the study of ant olfactory systems and their role in mediating that biology. In doing so, our intent is to go beyond an simple accounting of these efforts to highlight several avenues for future studies that will address critical knowledge gaps to provide a better understanding of the fundamental aspects of eusocial insect biology.

The Peripheral Olfactory System

The complex array of sensory neurons and support cells that together make up the peripheral olfactory system represent the initial site of chemical detection in ants. Here, pheromones, karimones, and other semiochemicals are detected by an array of membrane-bound chemoreceptors expressed in discrete suites of olfactory sensory neurons (OSNs). The function of these OSNs rely on a spectrum of signal transduction pathways that comprise both extra- and intracellular components centered around three classes of transmembrane chemoreceptors: odorant receptors (ORs), gustatory receptors (GRs), and ionotropic receptors (IRs) (for a detailed discussion of these and other chemoreceptor proteins involved in Hymenopteran olfactory biology, we direct the reader to (Ferguson, Anand, and Zwiebel 2020)). In brief, individual ORs are expressed in odorant receptor neurons (ORNs) alongside the obligate and highly conserved

odorant receptor co-receptor (Orco) (Gao and Chess 1999; Clyne et al. 1999; Vosshall et al. 1999; Fox et al. 2001). The ORs are involved in the detection of pheromones and other general odorants which for ants notably include the CHCs (Slone et al. 2017; Pask et al. 2017). The IRs are derived from an independent lineage of chemoreceptors and are responsible for the detection of acids and aldehydes (Benton et al. 2009; Abuin et al. 2011; Croset et al. 2010). The GRs are the most ancient chemoreceptor family in insects and are responsible for the detection of tastants and carbon dioxide (Clyne, Warr, and Carlson 2000). Curiously, while empitical evidence suggests that ants are able to detect carbon dioxide (Kremer et al. 2018; Romer, Bollazzi, and Roces 2017; Kleineidam and Tautz 1996), they have lost the canonical CO_2 receptors found in dipteran species (Robertson and Kent 2009; Zhou et al. 2012). Beyond these primary chemoreceptor families, there are a number of other ancillary support proteins involved in olfactory signaling. These include odorant degradation enzymes (ODEs) and a variety of odorant binding proteins (OBPs) and chemosensory proteins (CSPs). The former, as their name implies, facilitate the degradation of odorants (Ishida and Leal 2005; Vogt and Riddiford 1981), while the function of the latter is not as well understood. However, it is commonly thought that the OBPs and CSPs may facilitate odor transport through the sensilla lymph (Vogt, Riddiford, and Prestwich 1985), although they are evidently not required for olfactory responsiveness (Larter, Sun, and Carlson 2016; Xiao, Sun, and Carlson 2019).

The OSNs subtend hair-like sensilla that are stereotypically distributed along the antennae and other chemosensory appendages (Pask and Ray 2016). For ants, there are several different types of sensilla that vary in function, innervation, and morphology (Nakanishi et al. 2009; McKenzie et al. 2016). These notably include basiconic (broadly chemoreceptive including CHCs), ampullaceal (putatively CO_2 receptive), chaetic (contact based chemosensation), coelocapitular (hygro- and thermoreceptive), coeloconic (chemoreceptive), and trichoid (chemoreceptive) sensilla. In contrast to the relatively simple Dipteran olfactory system, which may have only a handful of OSNs in each sensillum, ants sensilla may contain over 130 ORNs (Nakanishi et al. 2009). In addition, there are important sexual dimorphisms with respect to the broad morphology of the antennae and the composition of sensilla between female and male ants. The basiconic sensilla, which presumably house the OSNs involved in CHC detection (Ozaki et al. 2005; Sharma et al. 2015), are notably absent in males and likely reflect the distinct physiological function of behavior of the different members of the colony (Esslen and Kaissling 1976; Nishino et al. 2009; Nakanishi et al. 2009). Altogether, the peripheral olfactory system in

ants shares many features in common with other insect species; however, evolution has produced an unparalleled level of complexity in ants that is unrivaled even by their honeybee and wasp counterparts.

Untangling Odor Coding in the Peripheral Sensilla

While there is a rich body of literature describing the source and function of pheromones and other semiochemicals that regulate the collective social behaviors in ant colonies, considerably less is known about odor coding in the antennae and other olfactory appendages. One area of particular interest is the characterization of the olfactory proceses involved in translating the CHC signatures that underlie nestmate recognition whereby conspecific ants are able to discriminate workers from their home colony (nestmates) which are met passively as "friends" from workers from other colonies (non-nestmates) which usually are treated aggressively as "foes". This has proven to be exceptionally challenging to address due to the complex CHC blends that are utilized along with the combinatorial and multifaceted nature of CHC detection. At first glance, the CHC profiles of conspecific workers from different colonies are often qualitatively similar, differing only in the subtle quantitative differences in the proportion of a given hydrocarbon (Guillem, Drijfhout, and Martin 2016; Martin, Helanterä, and Drijfhout 2008; Martin, Helantera, and Drijfhout 2008; Sharma et al. 2015). Despite these seemingly imperceptible differences, many species of ants are robustly able to use this information to distinguish friends from foes (Guerrieri et al. 2009), identify the task of a fellow nestmates (Greene and Gordon 2003), and discriminate aberrant worker-laid eggs from those of their queen (Endler et al. 2004). This remarkable sensory accuity is accomplished, at least in part, through CHC detection by the multiporous basiconic sensilla (Sharma et al. 2015). The odor coding in the ant basiconic sensilla remains enigmatic due to the astonishingly high number of OSNs (>130) which are present (Nakanishi et al. 2009) and that may be interacting with each other either directly via gap junctions (Takeichi et al. 2018) or indirectly via ephaptic transmission (Su et al. 2012). Furthermore, as of this writing, the precise composition of chemoreceptors expressed by these diverse OSNs remains unknown, although there are at least three subtypes of basiconic sensilla in two *Camponotus* species that collectively detect more than 10 general odorants and at least 20 different hydrocarbons (Sharma et al. 2015). Taken together, the sheer diversity of stimuli as well as the range of interacting neuronal and

molecular receptors represents a profoundly complex odor coding process that is likely to be beyond our understanding for quite some time to come.

While challenging, deciphering at least some of the linkage between the subtle complexity with which information is encoded in CHC profiles and the densely packed OSNs in the basiconic sensilla will undoubtedly represent a substantial milestone in olfactory myrmecology. To that end, several conflicting hypotheses have been proposed and experimentally examined. Single sensillum recordings (SSRs) showing that *C. japonicas* workers only respond to non-nestmate (but not nestmate) CHC blends, lead to the suggestion that ants may be anosmic to their own colony odor (Ozaki et al. 2005). If this effect were broadly observed, this would be remarkable because the hydrocarbons comprising nestmate and non-nestmate CHC blends are presumably the same, differing only in their ratio (Guillem, Drijfhout, and Martin 2016; Martin, Helanterä, and Drijfhout 2008; Martin, Helantera, and Drijfhout 2008; Sharma et al. 2015). However, subsequent studies using both SSR and antennal lobe activity imaging have not replicated these findings as both nestmate and non-nestmate CHCs were detected in the antennae and antennal lobe glomeruli, respectively (Brandstaetter AS 2011; Brandstaetter 2011; Sharma et al. 2015). A number of attempts have been made to reconcile these discordant findings. For example, it has been suggested, but as yet not validated experimentally, that there are at least two sensilla subtypes: one dedicated to detecting non-nestmate CHCs and another that detects a broad spectrum of hydrocarbons (Bos and d'Ettorre 2012). As things stand, we are left with more questions than answers, such that peripheral and central odor coding in eusocial insects remains largely hypothetical.

Identifying Odor Ligands through the Deorphanization of Chemoreceptors

Compared to the vast literature on ant pheromone biochemistry and chemical ecology, far less is known about the chemoreceptors involved in the detection of these odorants. Several efforts to functionally characterize (a process sometimes referred to as "deorphanization") ant chemoreceptors through the identification of their biologically salient odor ligands have been carried out. While initial deorphanization studies identified the receptor for 4-methoxyphenylacetone in *H. saltator* (HsOr55) and 2,4,5-trimethylthiazole in *C. floridanus* (CfOr263) (Zhou et al. 2012), the two most notable studies in this regard were conducted in *H. saltator* (Pask et al. 2017; Slone et al. 2017) where the electrophysiological responses of 47 ORs

across 9 different subclades were examined against a panel of synthetic and naturally obtained hydrocarbons and a range of other general odorants. These studies revealed that while the rapidly evolving 9-exon OR family is able to detect CHCs and therefore remains a compelling aspect of Hymenopteran olfaction, it is clear that pheromone detection is not limited to this subfamily. While several members of the large 9-exon subfamily—HsOr263, HsOr271, and HsOr259-L2—were indeed responsible for the detection of 13,23-dimethylheptatriacontane, a putative queen pheromone, responses to other hydrocarbons as well as a range of general odorants were broadly detected across the various subclades. These notably include a male enriched OR (HsOr36) from the L subfamily that responded to long chain alkanes, a worker enriched subfamily H receptor (Hs210), a subfamily V receptor (HsOr170), and a subfamily E receptor (HsOr236). These studies are important because they contribute to our understanding of the evolution of olfactory function in social insects (d'Ettorre, Deisig, and Sandoz 2017), yet these relatively modest efforts only scratch the surface. Future studies on the functional characterization of chemoreceptors in ants will strive to examine a broad range of taxa and use a significantly larger library of odorant stimuli, including but not limited to the hydrocarbons. Therefore, future efforts should also strive to incorporate receptors from non-9-exon and species-specific subclades. Given the diverse ecology and extensive chemoreceptor repertoire in ants, addressing this knowledge gap would be a monumental accomplishment. By extending these studies to other species, to other chemoreceptor families, and to the various subclades within each family, we will develop a better understanding of the evolution of eusociality, the molecular mechanisms involved in social behavior, and pave the way for future studies by identifying candidates for gene editing and other targeted molecular approaches.

Central Olfactory System

Parallels between the Insect and Vertebrate Olfactory System

At a cellular level, the fundamental organization of the olfactory system is remarkably similar across vertebrate and insect species (Hildebrand and Shepherd 1997; Strausfeld and Hirth 2013). Across this broad evolutionary distance, diverse OSNs residing in an aqueous milieu receive chemical messages from the environment and this information is relayed to the central brain via dedicated axonal tracts, converging on secondary neurons, local interneurons, and glial cells that together constitute the neuropil which forms the stereotypic glomeruli of the vertebrate olfactory bulb or the insect antennal lobe (AL) (Galizia and Sachse 2010; Wilson and Mainen

2006). Until recently it was doctrine that a single glomerulus was typically innervated by a specific corresponding set of peripheral OSNs, many of which express the same chemoreceptor (Couto, Alenius, and Dickson 2005; Fishilevich and Vosshall 2005). There may, however, be important exceptions to this rule as emerging studies from *Drosophila* and the yellow fever mosquito *Aedes aegypti* reveal that a single OSN may co-express receptors from different chemoreceptor families and are linked to multiple AL glomeruli (Younger et al. 2020; Task et al. 2020). In any case, having arrived at their respective (or collective) AL glomeruli, synaptic connections relay information to a collection of secondary glomerular neurons, known in insects as AL projection neurons, which are comparable to vertebrate olfactory bulb mitral and tufted cells. The initial processing of peripheral olfactory information that eventually leads to odorant discrimination and presumably perception occurs through the combinatorial activation of glomeruli that is transformed through integrative (often inhibitory) crosstalk between glomeruli via local interneurons (Christensen, Waldrop, and Hildebrand 1998; Sachse and Galizia 2002; Sachse et al. 2006; Olsen, Bhandawat, and Wilson 2007; Shang et al. 2007). Projection neurons subsequently connect the olfactory bulb or AL to the olfactory cortex and other central brain structures in vertebrates or, in the case of insects, to the mushroom bodies and lateral horn of the protocerebrum (Galizia and Sachse 2010; Wilson and Mainen 2006). In ants and other Hymenoptera, projection neurons are organized into a unique, dual olfactory pathway consisting of a medial and lateral output tract connecting to higher order brain structures which may improve olfactory information processing (Kirschner et al. 2006; Zube et al. 2008). These structures are then responsible for more complex cognitive processes. It has been suggested that insect mushroom bodies are responsible for learning and memory (Vowles 1964; Hammer and Menzel 1995; Erber, Masuhr, and Menzel 1980) whereas the lateral horn may play a role in learned and innate behavioral responses (Schultzhaus et al. 2017).

Structure and Function of the Antennal Lobe

While there are indeed many parallels between the insect and vertebrate olfactory system, there are also notable differences in terms of scale, structure, and function. Mice have well-over a thousand olfactory bulb glomeruli which, following the oft-cited, “one-receptor-one neuron-one glomerulus” rule (Maresh et al. 2008; Serizawa, Miyamichi, and Sakano 2004), derives from a correspondingly similar number of ORs (Royet et al. 1988; Buck and Axel 1991). In contrast,

Drosophila maintain only about sixty AL glomeruli (Stocker et al. 1990; Fishilevich and Vosshall 2005). As one might expect given their significantly larger OR repertoire, the complexity of ant ALs fall somewhere in-between—the clonal raider ant *Ooceraea biroi*, for example, has approximately 500 glomeruli (McKenzie et al. 2016). Importantly, the precise composition of the ant AL varies dramatically between different colony members within a given species with respect to age, task, and morphology (Brown, Napper, and Mercer 2004; Sigg, Thompson, and Mercer 1997; Arnold, Budharugsa, and Masson 1988; Mysore et al. 2009; Kelber C 2010). Previous experience and exposure to different environmental conditions may also lead to changes in glomerular volume, odor coding, and behavior (Jernigan et al. 2020). A distinct group of larger *Camponotus* workers ("majors") have a correspondingly larger glomerular volume but fewer glomeruli compared to minor workers (Mysore et al. 2009). By contrast, larger workers in the leaf-cutting ant *Atta vollenweideri* have a greater number of glomeruli (Kelber C 2010). Interestingly, macroglomeruli have also been identified in the larger worker caste of leaf-cutting ants, and these may be responsible for the detection of trail pheromones (Kleineidam et al. 2005). Furthermore, the ALs display profound sexual dimorphisms. In *Camponotus japonicas*, sterile female workers and virgin queens have roughly 430 glomeruli whereas the antennal lobe of males is reduced to only 215 glomeruli (Nishikawa et al. 2008). Males also have larger structures called macroglomeruli which are thought to be involved in the detection of sex-pheromones (Nakanishi et al. 2010; Nishikawa et al. 2008; Arnold, Masson, and Budharugsa 1985; Sandoz 2006; Nishino et al. 2009). These male-specific characteristics may reflect their marginalized role as short-lived reproductives. Overall, these changes likely reflect the unique behavioral and reproductive tasks carried out by different members of an ant colony.

Olfactory Sensory Neurons and the Ontogeny of the Antennal Lobe

Another notable difference between the insect and vertebrate olfactory system concerns the relationship between diverse sets of OSNs and the ontogeny of the AL glomeruli. For *Drosophila*, antennal lobe development occurs through three phases which begin at the start pupation when dendrites from second-order projection neurons arrive at stereotypic sites in the brain (Jefferis et al. 2004). In the second phase, OSN axons from peripheral olfactory appendages arrive at target sites in the proto-antennal lobe. This second phase notably occurs prior to OR gene expression, and not surprisingly, there are no significant structural alterations to the glomeruli of *orco* null

mutant *Drosophila* (Larsson et al. 2004). Furthermore, OSNs survive through development but degenerate later in adulthood. This is in contrast to mice and other mammals, where ORs are required for proper axon targeting (Lodovichi and Belluscio 2012). In the final phase, projection neurons and the axons from OSNs establish local synaptic connections to the exclusion of neighboring cells to create discrete glomeruli.

Arguably, the most compelling distinction about the olfactory system in ants was the recent observation that *orco* function is required for the proper development of the AL glomeruli (Yan et al. 2017; Trible et al. 2017). This rather unexpected difference in ant brain development was first described after the successful generation of two genetic mutant ant species. In these sister papers, CRISPR-Cas9 gene editing was used to target and knock down *orco* in the jumping ant *Harpegnathos saltator* (Yan et al. 2017) and the clonal raider *Ooceraea biroi* (Trible et al. 2017). In addition to a profound loss of olfactory sensitivity as well as the alteration of several behavioral phenotypes, orco mutant ants displayed significant reductions in both OSN population and the number and volume of AL glomeruli. More recently, antennal lobe development in *O. biroi* has been closely examined during the critical two-week pupation period (Ryba et al. 2020). In contrast with *Drosophila* (Larsson et al. 2004), OR expression occurs much earlier in ant development before the formation of glomeruli (Ryba et al. 2020). Indeed, Orco expression was high on the first day of pupation, and almost all of the nearly 500 ant ORs were expressed by day two of the pupal stage. Moreover, while *orco* is localized to the dendrites and cell bodies of fruit fly ORNs (Larsson et al. 2004), in the clonal raider ant, it is also found in ORN axons and axon terminals in the brain. Here, unilateral antennal ablations (that impact only the contralateral half of the bilaterally symmetric antennal lobes) on the first day of pupation resulted in significantly reduced glomeruli in adults. When antennae were ablated later in pupation, development was arrested, but any glomeruli that had already formed survive to adulthood. When antennae are ablated in adult callow workers, AL glomeruli remain for at least two weeks. Taken together, this suggests that *orco* mutants have impaired antennal lobe development due to OSN loss which are necessary for the formation of glomeruli but not their maintenance. Curiously, approximately 90 glomeruli survive both the ablation treatment and in the *orco* null mutant (Ryba et al. 2020; Trible et al. 2017). The authors suggest these remaining glomeruli are not associated with IRN, GRN or other non-ORN linked glomeruli but instead may be a more basic template upon which the remainder of the more complex antennal lobe forms. Ultimately, however, we are left with more questions than answers,

and these studies provide a fruitful avenue for future research. Of particular interest would be developing a topographical map of the antennal lobe in ants, as is being done in the honeybee. A better understanding of which glomeruli are responsible for detecting which odors might shed more light on the role of the mysterious 90 glomeruli that survive, if they have a function at all, and how their development may differ than the remainder of the antennal lobe.

Genomics, Evolution, and the Regulation of Chemosensory Genes

Over the past decade, considerable progress has been made towards understanding olfactory genomics in eusocial insects (Ferguson, Anand, and Zwiebel 2020). During this time, more than 50 Hymenopteran genomes have come online, and sequencing efforts for many more are currently underway (Branstetter et al. 2018; Favreau et al. 2018). One of the most notable scientific discoveries resulting from this ever-growing repository of genomic data was the identification of significant changes in the chemoreceptor families (Zhou et al. 2015; Zhou et al. 2012; Smith, Smith, et al. 2011; Robertson and Wanner 2006; Wurm et al. 2011; Oxley et al. 2014; Werren et al. 2010; Smith, Zimin, et al. 2011; Bonasio et al. 2012). Specifically, there has been a massive expansion of ORs through gene birth-and-death evolution across Apocrita that directly correlates to the degree of eusociality (Zhou et al. 2012; Zhou et al. 2015). Among these, ants boast the largest number of ORs. Genome sequencing across the evolutionarily basal suborder Symphyta, which is devoid of any eusocial species, has been considerably more limited. One bioinformatic study completed thus far in Symphyta has revealed that the genome of the solitary wheat stem sawfly *Cephus cinctus* has not undergone the same expansion of ORs as seen in Apocrita (Robertson, Waterhouse, et al. 2018). A notable exception to the eusocial-driven expansion of OR gene families is the genomes of several species of solitary wasps including *Nasonia vitripennis* and *Microplitis demolitor*, each of which have more ORs than that of the eusocial honeybee *Apis mellifera* (Robertson, Gadau, and Wanner 2010; Zhou et al. 2015). Looking at chemoreceptors beyond ORs, the genome of the dampwood termite *Zootermopsis nevadensis* has a greatly expanded family of IRs (Terrapon et al. 2014). Similarly, cockroaches have the largest number of chemoreceptors of any insect species described to date, with massive expansions of the IR and GR families (Robertson, Baits, et al. 2018). While insufficient to fully explain the macroevolution of eusociality, the expanded capacity to detect and communicate chemical information likely facilitated the acquisition of the broad range of social behaviors that

doubtlessly also provided an adaptive advantage across diverse environments. These early genomic studies in Hymenoptera provided a clear sense of direction for future research spurred by the concurrent development of molecular tools in eusocial insects.

Targeted Gene Editing in Formicidae

The first and arguably most significant Hymenopteran gene editing accomplishment thus far was carried out in Formicidae using CRISPR-Cas9 technology to knock out the Orco OR-coreceptor in two different species of ants, *Harpegnathos saltator* and *Ooceraea biroi* (Yan et al. 2017; Trible et al. 2017). In addition to the neuroanatomical effects discussed above, the authors of these two studies reported a number of physiological and behavioral deficits in *orco*$^{-/-}$ mutants that would likely impact eusociality. To begin with, in the absence of OR-mediated signaling, social cohesion within colonies was significantly diminished as workers wandered outside of the colony and neglected to engage in brood care. As to be expected, mutant workers displayed a loss of responsiveness to a number of olfactory cues and failed to follow trail pheromones or congregate with nestmates. Mutant workers from both species also had low fecundity. Taken together, these studies were meaningful not only because of the biological insights gleaned but also for their technical merit in extending gene-targeting to eusocial insects in spite of their unique reproductive division of labor.

The Technical Challenges of Gene Editing in Eusocial Hymenoptera

The toolbox available for examining the molecular biology of *Drosophila* as an academic model system has grown immensely since the pioneering genetic studies of Thomas Hunt Morgan at the turn of the 20th century. However, despite the availability of these resources, the transfer of these techniques to ants and other non-model insects has not been as rapid as many investigators had initially expected. For example, in comparison to the now countless numbers of mutant *Drosophila* lines that have been produced, there are (at this writing) only 3 published studies that have successfully utilized CRISPR-Cas9 or any other type of gene editing in ants (Yan et al. 2017; Trible et al. 2017; Chiu et al. 2020). This is not entirely surprising given the unique constraints imposed by the unique reproductive biology and other atypical features of many eusocial Hymenopteran relative to *Drosophila*.

To begin with, it is important to appreciate that, unlike the short and experimentally amenable lifecycle of solitary *Drosophila* and other Diptera (which often can be individually mated), the generation time in ant colonies can be quite long. Ant colonies are typically comprised of one or several extremely long-lived reproductive queens (Hölldobler and Wilson 1990). As the colony matures, diploid virgin queens and haploid males will emerge from the colony to engage in a mating flight before establishing a new colony. Even if an ant colony had a much shorter generation time and was capable of producing many offspring, the reproductive timing of virgin daughters and reproductive males is largely unknown (Hölldobler and Wilson 1990). In addition, ant colonies reared in a lab setting do not always produce reproductives, perhaps in part due to the use of temperature and humidity controlled incubation chambers (S.T. Ferguson, personal observation). Futhermore, it is currently not possible to identify the subset of embryos that will ultimately develop into reproductive queens and which will develop into sterile workers at the very narrowly timed syncytial blastoderm developmental stage required for robust CRISPR-Cas9 injections to target pole cells representing the inherited germlines. While a colony may produce hundreds of millions of eggs over its lifespan (Higashi and Yamauchi 1979), which at first might seem ideal for injection-based gene editing, the vast majority of these develop into sterile females. Therefore, as challenging as it may be, although the injection itself may be successful and yield a viable larval stage transgenic, it is extremely difficult to develop genetic lines let alone rear sufficient numbers of individuals for studies that involve the collective behavior of a full colony.

Given these challenges, successful gene editing in three ant species must be viewed as exceptional although it is noteworthy that each of these studies exploited a specific quirk of reproductive biology. For example, after the death of a queen, workers in *Harpegnathos saltator* colonies compete in a ritualized dueling behavior. The winner of these bouts undergoes a series physiological changes to become a reproductive gamergate. In the absence of nestmates, segregated workers will also transition to gamergates. Prior to mating, gamergates will lay eggs that develop into males. After mating, they are capable of producing female workers that continue to maintain the colony. Taking advantage of this, investigators designed guide RNAs (gRNAs) targeting the *H. saltator orco* gene that, together with Cas9 protein, were microinjected into male embryos. In order to prevent the destruction that typically occurs when manipulated embryos are reintroduced in *H. saltator* colonies, all injected embryos were independently reared outside the colony for 1 month on agar plates. Only after injected embryos had hatched into larvae were they

placed into small nests together with a limited number of helper workers that acted as nurses that were required for larval survival. Resultant adults were then outcrossed to produce a mix of mutant and wildtype male offspring (Yan et al. 2017). The genotype of these males was identified nonlethally by sequencing tissue samples obtained from the wing. Through a series of successive crosses extending over more than 1 year, mutant males were used to eventually establish homozygous mutant lines. The clonal raider ant *O. biroi* has a fundamentally different reproductive system characterized by queenless colonies, in which workers reproduce parthenogenetically. Here, mutant lines (also targeting *O. biroi orco*) were established from injected individual embryos without the need for extensive crosses (Trible et al. 2017). More recently, CRISPR-Cas9 has been used to induce somatic mutations in the fire ant, *Solenopsis invicta* (Chiu et al. 2020). Here, rather than attempt to generate stable mutant lines, the authors directly injected worker embryos with Cas9 together with gRNAs targeting *GP-9*, which encodes an odorant binding protein suspected of being associated with colony form, and *Sinv-spitz*, which was thought to be involved in establishing larval oenocytes. While these investigators used PCR to successfully establish some molecular evidence of gene-targeting they were unable to observe any physiological or behavioral phenotypes (Chiu et al. 2020). At the end of the day, the absence of phenotypic effects raises questions as to the utility an individual level approach for examining the biology of eusocial ants that function within complex colonies that have often been described as "superorganisms" (Hölldobler and Wilson 2008).

Innovative Variations and Alternatives to Gene Editing

These gene editing studies highlight the ingenuity of the investigators, the creativity often required of scientific endeavors, and, in the case of *H. saltator* and *O. biroi*, have provided unique insights into the olfactory system and social behavior of ants. Without diminishing these accomplishments, one might raise a caveat in that the methods employed rely upon the decidedly atypical reproductive biology of *H. saltator* and *O. biroi*, both of which are not representative of most ant species. Therefore, it is reasonable to suggest the existential challenges associated with gene editing in eusocial species remain to be addressed in a more direct, generalizable way. One potential solution may be through an innovative approach to insect gene editing which has been termed "ReMOT Control", short for Receptor-Mediated Ovary Transduction of Cargo (Chaverra-Rodriguez et al. 2018). Here, CRISPR-Cas9/gRNA machinery is delivered to developing eggs

during vitellogenesis using modified yolk protein precursors that are transported from the hemolymph into the ovaries. Indeed, this method has proven successful across a broad range of insect species (Heu et al. 2020; Chaverra-Rodriguez et al. 2020; Macias et al. 2020). Another approach might be to deliver the CRISPR-Cas9/gRNA complex using transfected sperm, a protocol for which has been successfully developed in birds using a cationic-lipid based chemical transfectant (Cooper et al. 2017), by artificially inseminating virgin queens (den Boer, Boomsma, and Baer 2013).

While there is no argument that the generation of *orco*$^{-/-}$ mutants in *H. saltator* and *O. biroi* represented a quantum leap in olfaction studies in ants, an additional and often-overlooked consideration that is salient for gene editing studies in any system is the potential for off-target effects. Indeed, the catastrophic changes to the antennal lobe during development represent a nontrivial confounding variable. Taken together with more recent efforts (Ryba et al. 2020), these studies suggest that OR function plays a necessary role in a variety of social behaviors which contribute to the evolutionary success of these insects. That said, it is not possible to distingush whether the behavioral phenotypes observed in these mutants are the result of the loss of olfactory signaling from the antennae, the large defects in the antennal lobe, or any number of potential changes encountered during an altered developmental program.

To address this confounding factor, we recently took advantage of a set of recently identified, novel pharmacological agents that acutely and selectively modulate Orco activity to examine the role of OR signaling in nestmate recognition (Ferguson et al. 2020). These compounds include an allosteric agonist, and allosteric antagonist, as well as a physiologically and pharmacologically inert analog control which can be applied as volatiles to wildtype adult ants. This method provided a potentially superior alternative to genetic engineering in that it disrupts olfactory signaling at a discrete time point in wildtype adults that had a normal developmental trajectory and were not subject to nearly impossible-to-rule-out off-target pleiotropic effects. Administration of an Orco antagonist conclusively demonstrated that OR-signaling is necessary for eliciting aggression towards non-nestmates, and moreover that the lack of familiar nestmate signals is not sufficient to elicit aggression. Parallel studies with the Orco agonist indicated that a mismatch between an olfactory cue and an endogenous template for nestmate odor profiles is also not sufficient to elicit aggression. Instead, aggression towards non-nestmates requires the OR-dependent detection of a precise chemical trigger present on the cuticle of a non-nestmate foe.

Importantly, because Orco is highly conserved across insect species, this method can readily be applied to diverse ant taxa. However, the broad utility of this approach is limited by lack of similar pharmacological agents against other cellular targets.

Advances in Epigenetic Engineering

Beyond gene editing, there have been other major technical advances in genomic myrmecology. Most notably, innovations in epigenetics. Eukaryotic DNA is compacted into chromatin complexes by wrapping around histone protein nucleosome octamers which are then altered through histone post-translational modifications (hPTMs) that directly influence the regulation of gene expression by altering the structure and accessibility of the DNA-protein complex (Luger et al. 1997; Kouzarides 2007). Methylation of cytosine nucleobases within DNA may also regulate gene expression and result in static 'imprinting' that impacts discrete genes as well as entire chromosomes (Cedar and Bergman 2009). Importantly, these processes, along with other epigenetic modifications, play a central role in the regulation of olfactory gene expression. Lysine methylation on histone 3 in the fruit fly, for example, determines OR gene expression by silencing the expression of all but one receptor (Clowney et al. 2011; Magklara et al. 2011; Sim et al. 2012). The development of novel approaches to modify this "histone code" may therefore represent a significant advance in the study of olfaction in ants and other insects.

Artificially Induced Histone Modifications Dramatically Alter Ant Behavior

While the genome of an individual organism is static, the methylome may vary across cells, tissues, and organisms. Genome-wide studies have now broadly characterized DNA methylation patterns and histone modifications in *Camponotus floridanus* reproductive and morphological castes (Glastad, Hunt, and Goodisman 2015; Simola et al. 2013). DNA methylation mapping revealed surprisingly few distinctions between queens and workers and between majors and minors. Furthermore, in these studies, gene expression did not seem to strongly correlate with DNA methylation. However, major and minor workers exhibit caste-specific enrichment of hPTMs. Acetylation of lysine 27 of histone H3 proteins (H3K27ac), which is typically associated with transcriptional activation, correlates with caste-specific gene expression patterns. In particular, binding sites for histone acetyltransferase (HAT) and CREB binding protein (CBP)—that are both involved in histone acetylation and transcriptional activation—displayed the greatest

variation between castes. Therefore, hPTMs may play a critical role in establishing transcriptional differences between morphological castes and task groups.

Previous studies have demonstrated that, in *Camponotus floridanus*, minor workers carry out the majority of the foraging while majors forage very little (Simola et al. 2016). However, microinjection of young majors with a histone deacetylase inhibitor (HDACi) —a class of epigenetic modifying drug that fosters chromatin acetylation—engaged in robust, minor-like foraging activity. This effect was inhibited when young majors were co-injected with both an HDACi and a histone acetyltransferase (HAT) inhibitor that would be expected to have opposing effects (Simola et al. 2016). To probe further into the effects of increased histone acetylation, age-matched older major and minor workers were treated with HDACis resulting in robustly increased foraging that was otherwise largely restricted to minor workers. RNAseq analyses showed increased abundance of 252 HDACi-responsive genes, with H3K27ac increasing near CBP binding sites. Importantly, simultaneous treatment with CBP-specific inhibitor suppressed the HDACi-induced foraging and similarly blocked the increased abundance of most of the 252 genes. These results strongly indicate that, in HDACi-treated minors, foraging was enhanced by CBP-dependent H3K27ac modifications. This is particularly interesting given emerging relevance of CBP to learning and memory in mammals (including humans) (Barrett et al. 2011). Notably, foraging in older adult major workers did not increase after HDACi treatment. While young majors exhibited strong enhancement of foraging after HDACi injection, this was blocked by co-injection of HDACi and HATi, mirroring treatment effects in minor foraging. Strikingly, HDACi-induced foraging in majors was stable for up to 50 days after a single injection, whereas foraging remained silenced in majors co-injected with both HDACi & HATi over the same term. These results support the hypothesis that histone acetylation regulates the behavioral differences between major and minor foragers and non-foragers.

The use of small molecule histone modifying pharmacological compounds offers yet another powerful molecular tool for the epigenetic study of myrmecology. While these studies do not directly address the role of olfactory signaling in directing worker behavior, the observation that histone modifications play an important role in the modulation of insect OR gene expression (Clowney et al. 2011; Magklara et al. 2011; Sim et al. 2012) makes a strong case for its involvement. Therefore, future studies might explore the connection between the epigenetic

regulation of chemoreceptor gene expression and social behavior in ant colonies using these and other molecular techniques.

Conclusions

Beginning with the publication of the first two ant genomes (Bonasio et al. 2010), there has been considerable progress in the field of olfactory myrmecology. Here, we review several of these recent major advances. These studies serve to differentiate the complex olfactory system in ants from other, more traditional model systems such as the fruit fly and provide a foundation to explore novel hypotheses within the unique social context of the ant society.

At the level of the peripheral olfactory system, research efforts have largely been focused on odor coding of chemical cues involved in social behaviors such as CHC-mediated nestmate recognition (Ozaki et al. 2005; Brandstaetter AS 2011; Brandstaetter 2011; Sharma et al. 2015) in addition to the functional characterization ("deorphanization") of odorant receptors (Slone et al. 2017; Pask et al. 2017). These studies have helped to unify molecular olfaction research with a longstanding interests in social behavior and CHC pheromone biochemistry. However, our understanding of peripheral olfactory signaling in ants is far from complete. Contradictory evidence which has yet to be resolved has been put forth regarding the precise odor coding of nestmate recognition cues (Bos and d'Ettorre 2012), and deorphanization studies have largely been restricted to only a single species (*H. saltator*) (Slone et al. 2017; Pask et al. 2017).

At the same time, there have been major advances in gene editing and other targeted molecular approaches in ants that will provide an essential foundation for addressing many outstanding questions within the field. Most notably, CRISPR-Cas9 was recently used to generate *orco* null mutant lines in two different species of ant (Yan et al. 2017; Trible et al. 2017). Beyond traditional gene editing techniques, the application of pharmacological compounds have been successfully used to study the epigenetic and olfactory regulation of social behavior in ants (Simola et al. 2016; Gospocic et al. 2017; Ferguson et al. 2020). Unfortunately, these studies have also been relatively limited in scope, and gene editing remains challenging due to the complicated reproductive biology characteristic of many ant species. Nevertheless, these initial gene editing studies shed light on the importance of odorant receptor function in the cohesion of social colonies, and provided a glimpse into the complicated, mammalian-like ontogeny of the AL glomeruli in ants (Yan et al. 2017; Trible et al. 2017; Ryba et al. 2020; Duan and Volkan 2020).

Overall, while the function of the OSNs in ants is similar to *Drosophila* and other dipteran insects, the growing consensus is that development of the ant AL is more reminiscent of its mammalian counterpart, the olfactory bulb. In that light, it has been suggested that the requirements for navigating complicated social interactions drive the increased complexity of the olfactory system in ants and mice which may in turn also constrain the developmental trajectory of the antennal lobe (Duan and Volkan 2020). These results highlight the importance of incorporating a broader range of model organisms in scientific research, as eusocial ants may be more advantageous than *Drosophila* or other traditional model systems when studying brain development in this context. Beyond the quirks of AL development in ants, relatively less is known about the form and function of the olfactory system during development, especially in larvae when information about colony identity may be first formed.

Despite the considerable progress that has been made over the past decade, marked by a series of high impact publications that have received attention both in the scientific community and beyond, much work remains to be done. These efforts, while challenging, are exceptionally meaningful, and eusocial insects are quickly becoming tractable model systems with a growing repository of tools. Altogether, these studies provide support for the use of basic research as a means to uncover more fundamental principles in biology that transcend seemingly disparate taxa and may have important implications for our understanding of vertebrate biology, including humans.

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Appendix A. Chapter II supplementary information††

OR Line	No UAS	HsOr4	HsOr20	HsOr32	HsOr35	HsOr36	HsOr37	HsOr55	HsOr59	HsOr161	HsOr170	HsOr174	HsOr175	HsOr177	HsOr182	HsOr183	HsOr188	HsOr190	HsOr209	HsOr210	HsOr212	HsOr224	HsOr236	HsOr259-L2	HsOr271	HsOr378	
Subfamily		L	L	L	L	L	L	L	L	V	V	V	V	I	D	D	B	J	H	H	H	E	E	9-exon	9-exon	H	
Enrichment			●	●	●	●	●			●					●	●	●		●	●	●	○		●	●	●	
	No UAS	HsOr 4	HsOr 20	HsOr 32	HsOr 35	HsOr 36	HsOr 37	HsOr 55	HsOr 59	HsOr 161	HsOr 170	HsOr 174	HsOr 175	HsOr 177	HsOr 182	HsOr 183	HsOr 188	HsOr 190	HsOr 209	HsOr 210	HsOr 212	HsOr 224	HsOr 236	HsOr 259-L2	HsOr 271	HsOr 378	
C10	-6.00	-13.20	-1.80	-7.80	-13.60	7.83	-5.40	-7.50	-2.50	-0.20	-2.60	4.40	-4.60	-8.00	0.80	0.80	-4.40	5.80	3.60	4.60	-5.00	-6.40	5.00	-0.80	-2.40	1.60	C10
C11	-6.40	-7.00	-1.80	-4.00	3.00	7.67	0.40	-8.50	3.83	-2.40	-1.20	-8.40	-4.80	0.20	0.60	6.80	3.20	4.80	5.20	-4.40	-2.60	0.20	-0.80	-1.40	-3.20	5.60	C11
C12	-1.00	-13.80	3.00	-10.00	-2.20	16.50	-4.40	-0.25	0.50	-3.00	3.00	1.80	-2.00	5.40	-1.20	4.00	-0.40	17.80	-1.60	6.20	-9.40	-1.60	0.40	1.00	-9.80	7.20	C12
C13	3.20	-9.60	5.20	-13.40	-9.20	8.83	-8.20	-8.00	-3.33	-0.60	3.00	1.80	-2.40	-1.00	3.60	3.40	-1.20	6.60	2.60	4.40	-7.80	-6.20	-11.00	-4.80	0.20	-2.00	C13
C14	3.00	-16.60	4.60	-9.20	-5.60	7.50	-7.80	-7.75	4.67	0.20	-0.80	6.00	-3.40	4.40	-1.40	5.80	-2.00	-1.00	4.00	6.00	2.40	-4.80	-1.00	-6.80	-11.40	1.80	C14
C15	12.60	-9.60	4.00	-2.00	-15.60	10.17	0.00	-4.50	-5.67	-2.20	-9.60	2.00	-8.80	1.20	4.20	3.20	-3.40	7.80	-5.80	4.40	1.00	-6.00	-3.80	-4.80	-6.80	-1.80	C15
C16	-8.60	-1.60	2.60	-8.60	-12.20	2.33	-5.40	-8.50	1.67	-1.20	-9.40	1.40	-5.40	-1.00	1.60	10.40	5.40	9.80	2.20	8.00	-7.00	-7.80	2.00	-0.80	-1.40	2.60	C16
C17	0.40	-4.00	2.80	-13.00	-19.20	3.83	4.80	-3.00	1.83	-2.80	-0.20	3.00	-4.80	0.60	6.40	10.00	-2.40	1.40	-0.60	1.20	-0.60	-4.80	3.40	-1.80	-4.00	-0.60	C17
C18	2.80	-5.00	8.40	-6.80	-7.40	0.33	-3.00	-7.00	2.50	-0.80	-4.20	3.80	-0.60	-3.20	3.80	0.40	-2.60	8.60	3.20	1.60	7.20	8.00	2.80	-2.80	-13.40	-2.00	C18
C19	4.00	2.80	1.00	-2.40	-4.20	4.17	-0.80	-3.50	0.67	5.40	-3.40	-2.00	-11.80	0.00	6.80	5.80	7.80	9.60	-3.40	-1.80	-0.60	-1.60	3.40	7.20	-3.60	5.40	C19
C20	2.80	15.20	0.60	0.00	-10.00	14.17	14.80	-8.25	-3.17	0.00	4.40	6.00	-9.60	8.40	0.60	6.40	33.20	12.00	-4.80	3.20	3.80	-8.80	-2.00	15.00	-1.80	7.20	C20
C21	-0.80	8.00	2.80	-4.80	-11.60	10.83	-0.60	-5.50	-3.83	1.80	7.80	0.20	-1.20	12.00	3.60	-3.40	17.80	13.60	-1.00	2.20	-2.00	-18.80	2.20	-6.80	12.20	10.60	C21
C22	5.80	0.80	-1.20	-2.60	-6.40	18.00	18.80	-3.75	4.33	4.40	3.00	1.00	4.80	14.00	0.60	1.60	27.00	12.20	1.60	6.80	-0.40	-11.60	3.00	-0.20	-7.20	6.40	C22
C23	-0.20	4.40	-8.00	-9.40	-8.00	6.83	13.20	-3.75	-4.00	2.20	6.00	9.00	-0.20	14.80	7.80	-3.40	15.40	13.40	-1.60	8.60	4.00	-3.20	-2.60	3.80	-4.60	2.60	C23
C24	-13.60	-3.00	5.60	-8.40	-8.40	10.83	8.00	-6.75	-4.83	-2.40	-3.00	6.80	1.60	9.40	12.20	5.40	-11.40	10.40	-1.60	-1.40	27.40	-5.20	3.60	-5.60	0.60	3.00	C24
C25	-2.20	5.80	5.40	-6.40	-8.60	11.67	22.80	-8.50	-5.67	-1.00	-2.00	3.80	-2.80	10.00	7.20	7.60	15.00	18.00	-2.80	6.20	18.00	-10.80	-2.00	2.20	-0.80	8.60	C25
C26	12.60	-8.20	3.00	-13.20	-11.80	15.83	12.60	-7.75	-2.67	0.60	10.00	2.40	2.40	11.00	5.40	6.00	15.40	14.80	2.80	5.80	6.20	-9.00	-6.20	-2.00	-8.40	-2.20	C26
C27	-1.80	8.00	5.60	-6.80	4.20	8.33	4.00	-1.25	-4.00	0.40	19.80	4.40	3.40	5.80	19.60	-5.60	15.20	16.40	-4.20	12.60	13.00	-9.40	-0.60	5.80	5.80	3.20	C27
C28	-7.60	-0.80	3.00	-2.00	2.20	41.83	15.80	-7.25	-6.17	-2.40	5.60	7.40	0.80	9.60	6.00	11.40	8.40	22.00	14.40	19.20	4.00	-4.20	-4.00	-0.60	1.00	5.20	C28
C29	2.60	6.20	0.40	0.40	-2.40	12.50	13.40	-3.00	-3.83	0.20	13.20	2.20	-5.60	16.40	14.80	7.40	27.40	19.80	6.20	11.60	17.00	-0.80	4.60	5.00	5.40	8.40	C29
C30	1.00	9.80	3.80	-2.80	22.40	9.67	9.60	-4.25	-1.00	-2.00	9.40	5.60	-3.40	19.60	12.20	-4.80	22.00	10.60	1.00	20.00	23.80	-10.80	-1.20	3.60	7.80	8.40	C30
C31	-1.80	19.40	12.80	-7.40	-1.80	11.00	13.20	-1.75	-3.17	0.60	12.20	9.20	-8.00	10.60	18.00	8.80	30.80	19.40	6.80	37.20	29.20	4.20	6.40	3.40	19.40	11.20	C31
C32	7.80	21.00	16.60	-7.00	24.20	13.67	15.80	-4.00	17.00	-0.40	17.20	7.00	5.60	24.00	17.40	4.20	20.40	38.40	14.60	41.20	24.20	-23.20	47.40	5.00	11.40	13.20	C32
C33	5.20	13.00	25.80	-4.60	-7.40	22.17	13.40	-6.75	7.50	2.40	32.40	-6.60	8.80	11.40	14.40	-5.00	27.40	12.00	0.40	15.80	18.00	-1.80	14.00	4.00	9.00	5.40	C33
C34	1.80	4.60	10.20	-9.20	6.00	19.50	10.60	-11.75	2.00	-3.00	17.40	7.40	-6.00	5.60	8.40	-1.00	31.80	13.20	7.20	15.20	1.80	-23.00	17.80	4.00	11.00	2.40	C34
C35	-1.00	16.80	18.20	2.80	3.00	20.17	16.40	-10.25	0.50	2.40	42.00	8.40	11.40	33.60	29.60	-1.60	24.60	24.00	18.60	15.00	34.00	-5.60	24.20	5.60	-5.60	9.80	C35
C36	3.60	9.40	22.20	-6.60	7.20	28.50	22.80	-5.50	2.00	9.20	30.80	5.80	-0.80	8.00	12.00	7.60	18.40	20.00	7.20	9.80	14.60	8.80	53.00	19.20	10.20	16.80	C36
C37	6.00	16.00	4.00	0.40	10.40	9.00	13.00	4.00	1.67	3.00	34.60	7.80	13.80	12.60	22.40	7.40	35.20	21.80	10.60	18.60	36.20	28.00	8.20	36.60	7.60	2.60	C37
C7-C40	0.20	-7.40	-4.40	-15.80	-13.00	13.67	11.80	-5.25	-0.83	0.20	1.20	2.80	1.80	-0.20	2.20	-10.00	10.20	9.00	5.40	-8.60	-10.40	-8.40	-0.20	7.00	-8.40	5.20	C7-C40
	No UAS	HsOr 4	HsOr 20	HsOr 32	HsOr 35	HsOr 36	HsOr 37	HsOr 55	HsOr 59	HsOr 161	HsOr 170	HsOr 174	HsOr 175	HsOr 177	HsOr 182	HsOr 183	HsOr 188	HsOr 190	HsOr 209	HsOr 210	HsOr 212	HsOr 224	HsOr 236	HsOr 259-L2	HsOr 271	HsOr 378	

Subfamily		L	L	L	L	L	L	L	L	V	V	V	V	I	D	D	B	J	H	H	H	E	E	9-exon	9-exon	H	
Enrichment			●	●	●	●	●			●					●	●	●		●	●	●	○		●	●	●	
	No UAS	HsOr	HsOr	HsOr	HsOr	HsOr	HsOr	HsOr	HsOr	HsOr	HsOr	HsOr	HsOr	HsOr	HsOr	HsOr	HsOr	HsOr	HsOr	HsOr	HsOr	HsOr	HsOr	HsOr	HsOr	HsOr	
C10	0.00	-7.20	4.20	-1.80	-7.60	13.83	0.60	-1.50	3.50	5.80	3.40	10.40	1.40	-2.00	6.80	6.80	1.60	11.80	9.60	10.60	1.00	-0.40	11.00	5.20	3.60	7.60	C10
C11	0.00	-0.60	4.60	2.40	9.40	14.07	6.80	-2.10	10.23	4.00	5.20	-2.00	1.60	6.60	7.00	13.20	9.60	11.20	11.60	2.00	3.80	6.60	5.60	5.00	3.20	12.00	C11
C12	0.00	-12.80	4.00	-9.00	-1.20	17.50	-3.40	0.75	1.50	-2.00	4.00	2.80	-1.00	6.40	-0.20	5.00	0.60	18.80	-0.60	7.20	-8.40	-0.60	1.40	2.00	-8.80	8.20	C12
C13	0.00	-12.80	2.00	-16.60	-12.40	5.63	-11.40	-11.20	-6.53	-3.80	-0.20	-1.40	-5.60	-4.20	0.40	0.20	-4.40	3.40	-0.60	1.20	-11.00	-9.40	-14.20	-8.00	-3.00	-5.20	C13
C14	0.00	-19.60	1.60	-12.20	-8.60	4.50	-10.80	-10.75	1.67	-2.80	-3.80	3.00	-6.40	1.40	-4.40	2.80	-5.00	-4.00	1.00	3.00	-0.60	-7.80	-4.00	-9.80	-14.40	-1.20	C14
C15	0.00	-22.20	-8.60	-14.60	-28.20	-2.43	-12.60	-17.10	-18.27	-14.80	-22.20	-10.60	-21.40	-11.40	-8.40	-9.40	-16.00	-4.80	-18.40	-8.20	-11.60	-18.60	-16.40	-17.40	-19.40	-14.40	C15
C16	0.00	7.00	11.20	0.00	-3.60	10.93	3.20	0.10	10.27	7.40	-0.80	10.00	3.20	7.60	10.20	19.00	14.00	18.40	10.80	16.60	1.60	0.80	10.60	7.80	7.20	11.20	C16
C17	0.00	-4.40	2.40	-13.40	-19.60	3.43	4.40	-3.40	1.43	-3.20	-0.60	2.60	-5.20	0.20	6.00	9.60	-2.80	1.00	-1.00	0.80	-1.00	-5.20	3.00	-2.20	-4.40	-1.00	C17
C18	0.00	-7.80	5.60	-9.60	-10.20	-2.47	-5.80	-9.80	-0.30	-3.60	-7.00	1.00	-3.40	-6.00	1.00	-2.40	-5.40	5.80	0.40	-1.20	4.40	5.20	0.00	-5.60	-16.20	-4.80	C18
C19	0.00	-1.20	-3.00	-6.40	-8.20	0.17	-4.80	-7.50	-3.33	1.40	-7.40	-6.00	-15.80	-4.00	2.80	1.80	3.80	5.60	-7.40	-5.80	-4.60	-5.60	-0.60	3.20	-7.60	1.40	C19
C20	0.00	12.40	-2.20	-2.80	-12.80	11.37	12.00	-11.05	-5.97	-2.80	1.60	3.20	-12.40	5.60	-2.20	3.60	30.40	9.20	-7.60	0.40	1.00	-11.60	-4.80	12.20	-4.60	4.40	C20
C21	0.00	8.80	3.60	-4.00	-10.80	11.63	0.20	-4.70	-3.03	2.60	8.60	1.00	-0.40	12.80	4.40	-2.60	18.60	14.40	-0.20	3.00	-1.20	-18.00	3.00	-6.00	13.00	11.40	C21
C22	0.00	-5.00	-7.00	-8.40	-12.20	12.20	13.00	-9.55	-1.47	-1.40	-2.80	-4.80	-1.00	8.20	-5.20	-4.20	21.20	6.40	-4.20	1.00	-6.20	-17.40	-2.80	-6.00	-13.00	0.60	C22
C23	0.00	4.60	-7.80	-9.20	-7.80	7.03	13.40	-3.55	-3.80	2.40	6.20	9.20	0.00	15.00	8.00	-3.20	15.60	13.60	-1.40	8.80	4.20	-3.00	-2.40	4.00	-4.40	2.80	C23
C24	0.00	10.60	19.20	5.20	5.20	24.43	21.60	6.85	8.77	11.20	10.60	20.40	15.20	23.00	25.80	19.00	2.20	24.00	12.00	12.20	41.00	8.40	17.20	8.00	14.20	16.60	C24
C25	0.00	8.00	7.60	-4.20	-6.40	13.87	25.00	-6.30	-3.47	1.20	0.20	6.00	-0.60	12.20	9.40	9.80	17.20	20.20	-0.60	8.40	20.20	-8.60	0.20	4.40	1.40	10.80	C25
C26	0.00	-20.80	-9.60	-25.80	-24.40	3.23	0.00	-20.35	-15.27	-12.00	-2.60	-10.20	-10.20	-1.60	-7.20	-6.60	2.80	2.20	-9.80	-6.80	-6.40	-21.60	-18.80	-14.60	-21.00	-14.80	C26
C27	0.00	9.80	7.40	-5.00	6.00	10.13	5.80	0.55	-2.20	2.20	21.60	6.20	5.20	7.60	21.40	-3.80	17.00	18.20	-2.40	14.40	14.80	-7.60	1.20	7.60	7.60	5.00	C27
C28	0.00	6.80	10.60	5.60	9.80	49.43	23.40	0.35	1.43	5.20	13.20	15.00	8.40	17.20	13.60	19.00	16.00	29.60	22.00	26.80	11.60	3.40	3.60	7.00	8.60	12.80	C28
C29	0.00	3.60	-2.20	-2.20	-5.00	9.90	10.80	-5.60	-6.43	-2.40	10.60	-0.40	-8.20	13.80	12.20	4.80	24.80	17.20	3.60	9.00	14.40	-3.40	2.00	2.40	2.80	5.80	C29
C30	0.00	8.80	2.80	-3.80	21.40	8.67	8.60	-5.25	-2.00	-3.00	8.40	4.60	-4.40	18.60	11.20	-5.80	21.00	9.60	0.00	19.00	22.80	-11.80	-2.20	2.60	6.80	7.40	C30
C31	0.00	21.20	14.60	-5.60	0.00	12.80	15.00	0.05	-1.37	2.40	14.00	11.00	-6.20	12.40	19.80	10.60	32.60	21.20	8.60	39.00	31.00	6.00	8.20	5.20	21.20	13.00	C31
C32	0.00	13.20	8.80	-14.80	16.40	5.87	8.00	-11.80	9.20	-8.20	9.40	-0.80	-2.20	16.20	9.60	-3.60	12.60	30.60	6.80	33.40	16.40	-31.00	39.60	-2.80	3.60	5.40	C32
C33	0.00	7.80	20.60	-9.80	-12.60	16.97	8.20	-11.95	2.30	-2.80	27.20	-11.80	3.60	6.20	9.20	-10.20	22.20	6.80	-4.80	10.60	12.80	-7.00	8.80	-1.20	3.80	0.20	C33
C34	0.00	2.80	8.40	-11.00	4.20	17.70	8.80	-13.55	0.20	-4.80	15.60	5.60	-7.80	3.80	6.60	-2.80	30.00	11.40	5.40	13.40	0.00	-24.80	16.00	2.20	9.20	0.60	C34
C35	0.00	17.80	19.20	3.80	4.00	21.17	17.40	-9.25	1.50	3.40	43.00	9.40	12.40	34.60	30.60	-0.60	25.60	25.00	19.60	16.00	35.00	-4.60	25.20	6.60	-4.60	10.80	C35
C36	0.00	5.80	18.60	-10.20	3.60	24.90	19.20	-9.10	-1.60	5.60	27.20	2.20	-4.40	4.40	8.40	4.00	14.80	16.40	3.60	6.20	11.00	5.20	49.40	15.60	6.60	13.20	C36
C37	0.00	10.00	-2.00	-5.60	4.40	3.00	7.00	-2.00	-4.33	-3.00	28.60	1.80	7.80	6.60	16.40	1.40	29.20	15.80	4.60	12.60	30.20	22.00	2.20	30.60	1.60	-3.40	C37
C7-C40	0.00	-7.60	-4.60	-16.00	-13.20	13.47	11.60	-5.45	-1.03	0.00	1.00	2.60	1.60	-0.40	2.00	-10.20	10.00	8.80	5.20	-8.80	-10.60	-8.60	-0.40	6.80	-8.60	5.00	C7-C40
	No UAS	HsOr 4	HsOr 20	HsOr 32	HsOr 35	HsOr 36	HsOr 37	HsOr 55	HsOr 59	HsOr 161	HsOr 170	HsOr 174	HsOr 175	HsOr 177	HsOr 182	HsOr 183	HsOr 188	HsOr 190	HsOr 209	HsOr 210	HsOr 212	HsOr 224	HsOr 236	HsOr 259-L2	HsOr 271	HsOr 378	

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Subfamily		L	L	L	L	L	L	L	L	V	V	V	V	I	D	D	B	J	H	H	H	E	E	9-exon	9-exon	H	
Enrichment			●	●	●	●	●			●					●	●	●		●	●	●	●			●		
	No UAS	HSOr 4	HSOr 20	HSOr 32	HSOr 35	HSOr 36	HSOr 37	HSOr 55	HSOr 59	HSOr 161	HSOr 170	HSOr 174	HSOr 175	HSOr 177	HSOr 182	HSOr 183	HSOr 188	HSOr 190	HSOr 209	HSOr 210	HSOr 212	HSOr 224	HSOr 236	HSOr 259-L2	HSOr 271	HSOr 378	
Formic acid	104.83	102.00	80.13	49.67	50.67	32.25	33.17	61.86	121.00	31.83	44.50	47.33	75.17	102.00	54.00	42.33	124.40	90.67	70.60	56.40	102.60	51.17	78.50	128.20	100.33	69.50	Formic acid
Geraniol	216.50	193.00	220.63	171.33	172.67	134.75	237.83	212.57	268.50	107.00	177.00	179.83	144.67	252.20	185.60	137.17	241.00	159.33	165.20	173.80	214.00	200.33	143.17	217.00	162.00	162.33	Geraniol
Citronellol	196.00	162.17	205.88	162.33	148.00	132.00	194.67	197.57	242.33	91.17	155.83	164.00	146.33	216.80	134.20	99.33	181.60	142.67	162.40	145.80	188.60	216.83	141.17	203.00	156.83	154.00	Citronellol
Farnesol	174.33	131.83	149.13	131.33	106.00	109.25	150.50	182.57	179.00	115.67	135.17	129.83	143.17	165.40	122.20	122.50	150.60	127.50	149.20	118.20	131.20	148.33	120.33	141.60	137.83	146.50	Farnesol
Menthol	184.50	138.33	170.25	156.17	118.00	138.25	178.17	202.00	179.83	78.00	124.17	136.83	129.67	145.80	99.80	91.33	136.60	133.67	113.60	101.80	129.80	182.67	141.17	120.60	137.67	143.67	Menthol
Geosmin	129.17	120.50	131.00	112.83	113.83	104.75	108.50	131.00	133.83	141.00	131.83	104.00	120.00	110.60	97.20	124.00	137.40	114.17	123.20	112.00	103.40	134.33	125.83	111.20	123.33	110.50	Geosmin
4M3H	318.83	217.67	290.25	245.83	230.50	185.75	230.00	311.00	213.67	168.17	228.67	215.67	211.17	199.20	170.40	150.83	243.00	201.83	190.80	184.20	219.00	281.67	195.67	196.40	202.83	199.17	4M3H
Pentanol	571.17	312.33	424.38	253.50	340.17	179.50	259.50	507.43	336.67	537.33	383.00	396.00	306.00	275.20	302.80	346.50	421.60	280.17	424.00	307.20	290.00	324.17	237.00	383.20	314.17	247.67	Pentanol
3M1B	388.17	156.00	326.50	182.33	182.50	120.00	162.00	330.14	162.67	187.33	209.83	213.67	180.00	123.60	162.20	168.50	227.80	158.83	107.80	164.20	138.40	219.33	143.17	212.20	182.50	139.33	3M1B
1-Octen-3-ol	360.50	253.00	359.38	273.33	300.67	221.00	241.17	351.71	220.33	229.17	318.67	316.83	240.67	220.00	214.20	195.67	277.40	228.00	243.20	210.80	207.00	252.00	214.83	236.80	247.83	250.17	1-Octen-3-ol
Terpineol	258.00	234.33	273.50	259.83	261.83	207.50	272.67	337.29	175.83	162.00	218.67	276.17	224.50	183.00	177.20	185.50	224.80	213.00	160.20	161.60	159.40	255.67	223.00	176.00	220.00	278.83	Terpineol
Citral	201.17	205.33	238.63	223.00	225.33	160.75	208.83	237.43	158.33	108.83	172.17	207.50	180.17	170.80	182.80	207.17	225.20	162.33	175.00	158.40	166.60	210.00	167.17	211.40	172.17	198.33	Citral
E2-Hexenal	275.50	211.50	299.13	261.17	248.67	184.00	237.67	378.86	225.83	332.17	237.50	322.83	230.67	229.80	219.00	233.17	279.80	193.17	224.40	213.00	204.20	227.17	175.17	286.40	209.67	268.67	E2-Hexenal
Et. Isovalerate	232.83	189.67	244.50	170.33	185.00	193.25	192.33	229.57	244.00	316.50	241.83	259.50	170.67	232.00	236.60	184.83	209.20	181.83	270.20	198.80	211.80	209.50	163.33	253.40	167.33	234.00	Et. Isovalerate
Ger. Acetate	230.00	207.67	276.38	257.00	255.00	163.00	232.67	276.29	236.67	152.50	225.50	281.67	227.50	237.00	239.80	231.33	238.00	195.33	228.80	221.40	209.20	217.83	186.17	253.00	205.17	211.00	Ger. Acetate
M. salic	148.67	160.50	173.63	192.83	179.83	123.00	163.83	183.43	122.17	187.50	157.67	176.50	137.50	141.00	145.20	160.83	141.00	132.67	118.20	121.20	130.20	165.33	141.00	119.60	128.67	180.67	M. salic
Dod. Acetate	133.00	132.50	123.50	157.00	140.67	98.75	124.67	131.43	128.50	108.67	130.00	126.33	121.83	119.80	128.80	182.33	128.20	121.50	123.20	118.60	117.00	132.67	119.50	124.40	131.50	123.50	Dod. Acetate
Veratrole	139.17	119.00	133.50	154.33	158.50	106.00	137.67	157.57	133.17	134.33	138.33	135.17	110.83	128.40	136.80	176.00	119.20	118.67	122.00	116.40	121.60	129.33	124.00	124.60	118.00	136.67	Veratrole
Cineole	125.83	129.17	137.88	159.67	157.67	122.50	158.83	160.14	128.17	125.67	136.83	140.33	128.50	131.40	140.20	61.83	139.40	124.00	128.40	126.20	133.00	152.83	124.67	131.00	128.33	142.67	Cineole
Acetone	134.83	104.00	139.50	125.33	128.83	95.00	115.17	154.57	99.17	130.50	138.17	153.33	121.83	102.40	115.60	100.67	120.60	119.67	110.80	108.80	116.00	124.17	110.00	130.60	123.50	113.17	Acetone
Acetic Acid	204.83	138.17	181.25	89.50	104.17	17.25	31.33	186.29	99.67	28.17	30.17	251.83	54.83	121.20	95.40	99.50	143.20	78.83	123.00	64.00	133.60	88.00	84.17	230.60	167.33	87.50	Acetic Acid
EtOH	103.83	111.17	103.38	114.17	104.33	96.25	105.83	118.00	113.00	102.83	101.33	99.83	111.67	100.40	125.40	115.83	105.20	102.00	94.80	101.20	107.80	110.00	109.00	114.80	100.33	107.00	EtOH
Henkel 100	349.50	274.50	344.25	301.83	331.00	279.50	309.17	370.71	430.83	421.83	365.33	415.83	268.67	362.20	385.60	349.50	312.80	273.33	389.80	338.40	363.20	306.50	247.00	392.60	273.67	338.17	Henkel 100
Heptanone	358.67	245.83	326.25	262.83	319.50	258.50	338.83	391.00	412.00	361.67	327.00	412.83	261.00	343.80	362.80	298.83	308.40	282.00	409.60	320.20	341.00	301.17	228.33	363.20	246.17	370.83	Heptanone
Cyclohexanone	211.83	134.83	195.63	156.67	176.00	117.25	195.83	211.00	184.83	190.00	150.00	218.50	137.17	158.20	144.20	146.33	135.00	139.33	172.40	167.00	178.80	181.50	123.17	179.20	136.00	164.50	Cyclohexanone
4MPA	149.67	144.83	190.38	159.67	158.17	137.00	158.67	162.71	204.33	126.33	143.83	164.17	152.17	187.00	187.40	223.33	183.80	136.17	194.20	152.20	188.80	185.33	132.17	231.60	137.83	159.83	4MPA
6M5H	302.17	214.00	299.88	264.67	280.83	275.50	324.33	337.29	314.67	232.17	293.83	346.00	225.33	310.40	274.20	234.17	263.00	240.00	301.40	271.00	297.40	329.17	219.67	326.20	226.67	324.00	6M5H
DEET	107.50	123.67	120.13	128.50	129.50	97.50	107.67	124.29	76.83	126.33	103.17	124.67	110.50	98.00	112.80	137.50	117.80	106.00	84.60	102.60	93.40	113.50	112.33	94.20	121.17	114.00	DEET
Camphor	144.17	130.83	159.88	147.33	137.33	161.25	183.17	153.86	148.33	120.67	138.50	152.17	137.50	146.80	126.00	101.50	115.40	138.67	109.60	127.80	148.20	209.67	127.50	134.80	125.67	166.67	Camphor
2-nonanone	190.67	140.33	175.63	166.83	149.00	199.00	191.00	177.43	196.83	157.50	139.00	167.33	155.17	199.20	172.40	145.17	154.00	154.67	169.40	168.40	179.40	174.50	142.83	197.60	139.00	166.17	2-nonanone
Ethyl Acetate	263.50	188.50	279.00	196.00	208.50	156.00	165.83	227.00	283.67	451.33	256.50	386.17	219.00	205.40	209.20	304.67	184.20	185.17	258.80	225.20	212.20	197.67	185.33	281.40	220.50	232.50	Ethyl Acetate
2-Acetylthiophene	179.50	148.83	190.25	161.50	185.00	160.00	192.33	215.29	223.67	256.17	184.17	196.50	183.33	216.00	173.60	159.67	168.20	164.17	175.60	199.00	212.80	209.50	137.17	224.80	149.33	192.33	2-Acetylthiophene
Linalool	220.83	156.67	210.88	187.50	192.17	182.25	213.33	217.29	208.17	132.00	188.17	201.83	168.00	221.20	191.00	174.00	197.40	181.17	230.60	187.40	199.60	215.33	159.00	232.00	173.83	195.67	Linalool
3M1B	187.67	128.17	149.25	144.50	167.83	139.75	161.50	163.14	159.50	155.00	147.50	152.67	137.83	166.00	163.60	323.67	153.80	136.33	157.20	149.60	161.40	163.33	126.50	190.20	126.00	155.83	3M1B
2,4,5-TMT	224.17	218.33	281.75	181.33	208.00	127.50	144.00	210.14	321.67	272.33	266.33	246.50	208.33	267.20	254.20	301.33	244.20	189.00	377.20	189.40	264.00	145.17	200.00	305.00	230.17	221.50	2,4,5-TMT
2,3-Butanedione	205.50	197.17	205.13	150.67	205.83	187.00	165.00	216.29	199.67	310.50	224.83	220.83	167.83	220.20	177.40	172.67	192.80	170.33	208.80	174.40	181.20	201.00	161.17	206.20	168.83	185.83	2,3-Butanedione
Ethylpyrazine	182.67	196.83	231.00	195.17	227.83	186.50	205.50	199.14	232.00	222.00	250.17	209.67	213.67	243.40	251.80	308.50	181.40	186.17	256.80	240.00	222.60	209.50	173.17	273.60	223.17	190.17	Ethylpyrazine
Tetra-MP	217.50	159.17	205.38	162.17	180.50	150.50	146.67	224.43	189.67	265.17	191.33	219.00	191.50	198.20	188.60	221.17	158.20	162.50	212.60	167.80	191.40	181.50	148.67	240.00	179.67	157.50	Tetra-MP
2-M-Pyrazine	260.50	179.50	234.50	199.00	216.83	167.75	182.17	233.71	179.33	282.67	192.83	230.50	172.50	194.20	182.60	214.00	193.00	166.50	226.40	181.80	184.40	212.00	151.33	239.40	179.67	189.50	2-M-Pyrazine
2,6-DMP	136.67	105.83	108.63	115.67	111.83	104.25	106.33	124.57	113.00	133.50	122.17	99.67	99.83	112.60	118.00	125.17	102.80	109.00	110.40	108.00	115.20	92.83	102.17	127.20	107.83	99.83	2,6-DMP
Pyrazine	No UAS	HSOr 4	HSOr 20	HSOr 32	HSOr 35	HSOr 36	HSOr 37	HSOr 55	HSOr 59	HSOr 161	HSOr 170	HSOr 174	HSOr 175	HSOr 177	HSOr 182	HSOr 183	HSOr 188	HSOr 190	HSOr 209	HSOr 210	HSOr 212	HSOr 224	HSOr 236	HSOr 259-L2	HSOr 271	HSOr 378	Pyrazine

Subfamily		L	L	L	L	L	L	L	L	V	V	V	V	I	D	D	B	J	H	H	H	E	E	9-exon	9-exon	H	
Enrichment			●	●	●	●	●			●					●	●	●		●	●	●	●			●		
	No UAS	HSOr 4	HSOr 20	HSOr 32	HSOr 35	HSOr 36	HSOr 37	HSOr 55	HSOr 59	HSOr 161	HSOr 170	HSOr 174	HSOr 175	HSOr 177	HSOr 182	HSOr 183	HSOr 188	HSOr 190	HSOr 209	HSOr 210	HSOr 212	HSOr 224	HSOr 236	HSOr 259-L2	HSOr 271	HSOr 378	
Formic acid	1.00	0.97	0.76	0.47	0.48	0.31	0.32	0.59	1.15	0.30	0.42	0.45	0.72	0.97	0.52	0.40	1.19	0.86	0.67	0.54	0.98	0.49	0.75	1.22	0.96	0.66	Formic acid
Geraniol	1.00	0.89	1.02	0.79	0.80	0.62	1.10	0.98	1.24	0.49	0.82	0.83	0.67	1.16	0.86	0.63	1.11	0.74	0.76	0.80	0.99	0.93	0.66	1.00	0.75	0.75	Geraniol
Citronellol	1.00	0.83	1.05	0.83	0.76	0.67	0.99	1.01	1.24	0.47	0.80	0.84	0.75	1.11	0.68	0.51	0.93	0.73	0.83	0.74	0.96	1.11	0.72	1.04	0.80	0.79	Citronellol
Farnesol	1.00	0.76	0.86	0.75	0.61	0.63	0.86	1.05	1.03	0.66	0.78	0.74	0.82	0.95	0.70	0.70	0.86	0.73	0.86	0.68	0.75	0.85	0.69	0.81	0.79	0.84	Farnesol
Menthol	1.00	0.75	0.92	0.85	0.64	0.75	0.97	1.09	0.97	0.42	0.67	0.74	0.70	0.79	0.54	0.50	0.74	0.72	0.62	0.55	0.70	0.99	0.77	0.65	0.75	0.78	Menthol
Geosmin	1.00	0.93	1.01	0.87	0.88	0.81	0.84	1.01	1.04	1.09	1.02	0.81	0.93	0.86	0.75	0.96	1.06	0.88	0.95	0.87	0.80	1.04	0.97	0.86	0.95	0.86	Geosmin
4M3H	1.00	0.68	0.91	0.77	0.72	0.58	0.72	0.98	0.67	0.53	0.72	0.68	0.66	0.62	0.53	0.47	0.76	0.63	0.60	0.58	0.69	0.88	0.61	0.62	0.64	0.62	4M3H
Pentanol	1.00	0.55	0.74	0.44	0.60	0.31	0.45	0.89	0.59	0.94	0.67	0.69	0.54	0.48	0.53	0.61	0.74	0.49	0.74	0.54	0.51	0.57	0.41	0.67	0.55	0.43	Pentanol
3M1B	1.00	0.40	0.84	0.47	0.47	0.31	0.42	0.85	0.42	0.48	0.54	0.55	0.46	0.32	0.42	0.43	0.59	0.41	0.28	0.42	0.36	0.57	0.37	0.55	0.47	0.36	3M1B
1-Octen-3-ol	1.00	0.70	1.00	0.76	0.83	0.61	0.67	0.98	0.61	0.64	0.88	0.88	0.67	0.61	0.59	0.54	0.77	0.63	0.67	0.58	0.57	0.70	0.60	0.66	0.69	0.69	1-Octen-3-ol
Terpineol	1.00	0.91	1.06	1.01	1.01	0.80	1.06	1.31	0.68	0.63	0.85	1.07	0.87	0.71	0.69	0.72	0.87	0.83	0.62	0.63	0.62	0.99	0.86	0.68	0.85	1.08	Terpineol
Citral	1.00	1.02	1.19	1.11	1.12	0.80	1.04	1.18	0.79	0.54	0.86	1.03	0.90	0.85	0.91	1.03	1.12	0.81	0.87	0.79	0.83	1.04	0.83	1.05	0.86	0.99	Citral
E2-Hexenal	1.00	0.77	1.09	0.95	0.90	0.67	0.86	1.38	0.82	1.21	0.86	1.17	0.84	0.83	0.79	0.85	1.02	0.70	0.81	0.77	0.74	0.82	0.64	1.04	0.76	0.98	E2-Hexenal
Et. Isovalerate	1.00	0.81	1.05	0.73	0.79	0.83	0.83	0.99	1.05	1.36	1.04	1.11	0.73	1.00	1.02	0.79	0.90	0.78	1.16	0.85	0.91	0.90	0.70	1.09	0.72	1.01	Et. Isovalerate
Ger. Acetate	1.00	0.90	1.20	1.12	1.11	0.71	1.01	1.20	1.03	0.66	0.98	1.22	0.99	1.03	1.04	1.01	1.03	0.85	0.99	0.96	0.91	0.95	0.81	1.10	0.89	0.92	Ger. Acetate
M. salic	1.00	1.08	1.17	1.30	1.21	0.83	1.10	1.23	0.82	1.26	1.06	1.19	0.92	0.95	0.98	1.08	0.95	0.89	0.80	0.82	0.88	1.11	0.95	0.80	0.87	1.22	M. salic
Dod. Acetate	1.00	1.00	0.93	1.18	1.06	0.74	0.94	0.99	0.97	0.82	0.98	0.95	0.92	0.90	0.97	1.37	0.96	0.91	0.93	0.89	0.88	1.00	0.90	0.94	0.99	0.93	Dod. Acetate
Veratrole	1.00	0.86	0.96	1.11	1.14	0.76	0.99	1.13	0.96	0.97	0.99	0.97	0.80	0.92	0.98	1.26	0.86	0.85	0.88	0.84	0.87	0.93	0.89	0.90	0.85	0.98	Veratrole
Cineole	1.00	1.03	1.10	1.27	1.25	0.97	1.26	1.27	1.02	1.00	1.09	1.12	1.02	1.04	1.11	0.49	1.11	0.99	1.02	1.00	1.06	1.21	0.99	1.04	1.02	1.13	Cineole
Acetone	1.00	0.77	1.03	0.93	0.96	0.70	0.85	1.15	0.74	0.97	1.02	1.14	0.90	0.76	0.86	0.75	0.89	0.89	0.82	0.81	0.86	0.92	0.82	0.97	0.92	0.84	Acetone
Acetic Acid	1.00	0.67	0.88	0.44	0.51	0.08	0.15	0.91	0.49	0.14	0.15	1.23	0.27	0.59	0.47	0.49	0.70	0.38	0.60	0.31	0.65	0.43	0.41	1.13	0.82	0.43	Acetic Acid
EtOH	1.00	1.07	1.00	1.10	1.00	0.93	1.02	1.14	1.09	0.99	0.98	0.96	1.08	0.97	1.21	1.12	1.01	0.98	0.91	0.97	1.04	1.06	1.05	1.11	0.97	1.03	EtOH
Henkel 100	1.00	0.79	0.98	0.86	0.95	0.80	0.88	1.06	1.23	1.21	1.05	1.19	0.77	1.04	1.10	1.00	0.89	0.78	1.12	0.97	1.04	0.88	0.71	1.12	0.78	0.97	Henkel 100
Heptanone	1.00	0.69	0.91	0.73	0.89	0.72	0.94	1.09	1.15	1.01	0.91	1.15	0.73	0.96	1.01	0.83	0.86	0.79	1.14	0.89	0.95	0.84	0.64	1.01	0.69	1.03	Heptanone
Cyclohexanone	1.00	0.64	0.92	0.74	0.83	0.55	0.92	1.00	0.87	0.90	0.71	1.03	0.65	0.75	0.68	0.69	0.64	0.66	0.81	0.79	0.84	0.86	0.58	0.85	0.64	0.78	Cyclohexanone
4MPA	1.00	0.97	1.27	1.07	1.06	0.92	1.06	1.09	1.37	0.84	0.96	1.10	1.02	1.25	1.25	1.49	1.23	0.91	1.30	1.02	1.26	1.24	0.88	1.55	0.92	1.07	4MPA
6M5H	1.00	0.71	0.99	0.88	0.93	0.91	1.07	1.12	1.04	0.77	0.97	1.15	0.75	1.03	0.91	0.77	0.87	0.79	1.00	0.90	0.98	1.09	0.73	1.08	0.75	1.07	6M5H
DEET	1.00	1.15	1.12	1.20	1.20	0.91	1.00	1.16	0.71	1.18	0.96	1.16	1.03	0.91	1.05	1.28	1.10	0.99	0.79	0.95	0.87	1.06	1.04	0.88	1.13	1.06	DEET
Camphor	1.00	0.91	1.11	1.02	0.95	1.12	1.27	1.07	1.03	0.84	0.96	1.06	0.95	1.02	0.87	0.70	0.80	0.96	0.76	0.89	1.03	1.45	0.88	0.94	0.87	1.16	Camphor
2-nonanone	1.00	0.74	0.92	0.87	0.78	1.04	1.00	0.93	1.03	0.83	0.73	0.88	0.81	1.04	0.90	0.76	0.81	0.81	0.89	0.88	0.94	0.92	0.75	1.04	0.73	0.87	2-nonanone
Ethyl Acetate	1.00	0.72	1.06	0.74	0.79	0.59	0.63	0.86	1.08	1.71	0.97	1.47	0.83	0.78	0.79	1.16	0.70	0.70	0.98	0.85	0.81	0.75	0.70	1.07	0.84	0.88	Ethyl Acetate
2-Acetylthiophene	1.00	0.83	1.06	0.90	1.03	0.89	1.07	1.20	1.25	1.43	1.03	1.09	1.02	1.20	0.97	0.89	0.94	0.91	0.98	1.11	1.19	1.17	0.76	1.25	0.83	1.07	2-Acetylthiophene
Linalool	1.00	0.71	0.95	0.85	0.87	0.83	0.97	0.98	0.94	0.60	0.85	0.91	0.76	1.00	0.86	0.79	0.89	0.82	1.04	0.85	0.90	0.98	0.72	1.05	0.79	0.89	Linalool
2,4,5-TMT	1.00	0.68	0.80	0.77	0.89	0.74	0.86	0.87	0.85	0.83	0.79	0.81	0.73	0.88	0.87	1.72	0.82	0.73	0.84	0.80	0.86	0.87	0.67	1.01	0.67	0.83	2,4,5-TMT
2,3-Butanedione	1.00	0.97	1.26	0.81	0.93	0.57	0.64	0.94	1.43	1.11	1.19	1.10	0.93	1.19	1.13	1.34	0.99	0.84	1.68	0.84	1.18	0.65	0.89	1.36	1.03	0.99	2,3-Butanedione
Ethylpyrazine	1.00	0.96	1.00	0.73	1.00	0.91	0.80	1.05	0.97	1.51	1.09	1.07	0.82	1.07	0.86	0.84	0.94	0.83	1.02	0.85	0.88	0.98	0.78	1.00	0.82	0.90	Ethylpyrazine
Tetra-MP	1.00	1.08	1.26	1.07	1.25	1.02	1.12	1.09	1.27	1.22	1.37	1.15	1.17	1.33	1.38	1.69	0.99	1.02	1.41	1.31	1.22	1.15	0.95	1.50	1.22	1.04	Tetra-MP
2-M-Pyrazine	1.00	0.73	0.94	0.75	0.83	0.69	0.67	1.03	0.87	1.22	0.88	1.01	0.88	0.91	0.87	1.02	0.73	0.75	0.98	0.77	0.88	0.83	0.68	1.10	0.83	0.72	2-M-Pyrazine
2,6-DMP	1.00	0.69	0.90	0.76	0.83	0.64	0.70	0.90	0.69	1.09	0.74	0.88	0.66	0.75	0.70	0.82	0.74	0.64	0.87	0.70	0.71	0.81	0.58	0.92	0.69	0.73	2,6-DMP
Pyrazine	1.00	0.77	0.79	0.85	0.82	0.76	0.78	0.91	0.83	0.98	0.89	0.73	0.73	0.82	0.86	0.92	0.75	0.80	0.81	0.79	0.84	0.68	0.75	0.93	0.79	0.73	Pyrazine
	No UAS	HSOr 4	HSOr 20	HSOr 32	HSOr 35	HSOr 36	HSOr 37	HSOr 55	HSOr 59	HSOr 161	HSOr 170	HSOr 174	HSOr 175	HSOr 177	HSOr 182	HSOr 183	HSOr 188	HSOr 190	HSOr 209	HSOr 210	HSOr 212	HSOr 224	HSOr 236	HSOr 259-L2	HSOr 271	HSOr 378	

Figure A-1. Dataset S1.
Electrophysiological response values used to generate heatmap figures.

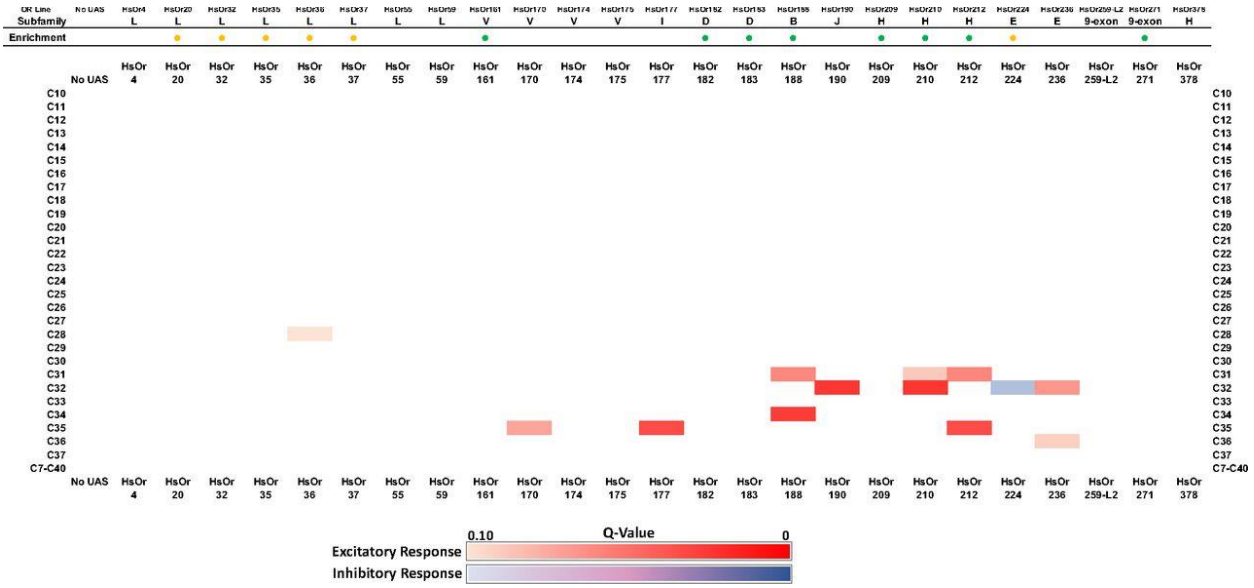

Figure A-2. Statistical analysis of SSR responses to CHCs. Analysis was conducted by using one-way ANOVA, followed by correction for multiple comparisons using a two-stage FDR method (Benjamini, Krieger, and Yekutieli 2006) with a threshold of q < 0.10.

Statistical analysis of SSR responses to CHCs. Analysis was conducted by using one-way ANOVA, followed by correction for multiple comparisons using a two-stage FDR method (Benjamini, Krieger, and Yekutieli 2006) with a threshold of $q < 0.10$. Significant excitatory responses are indicated in red, and inhibitory hits are indicated in blue.

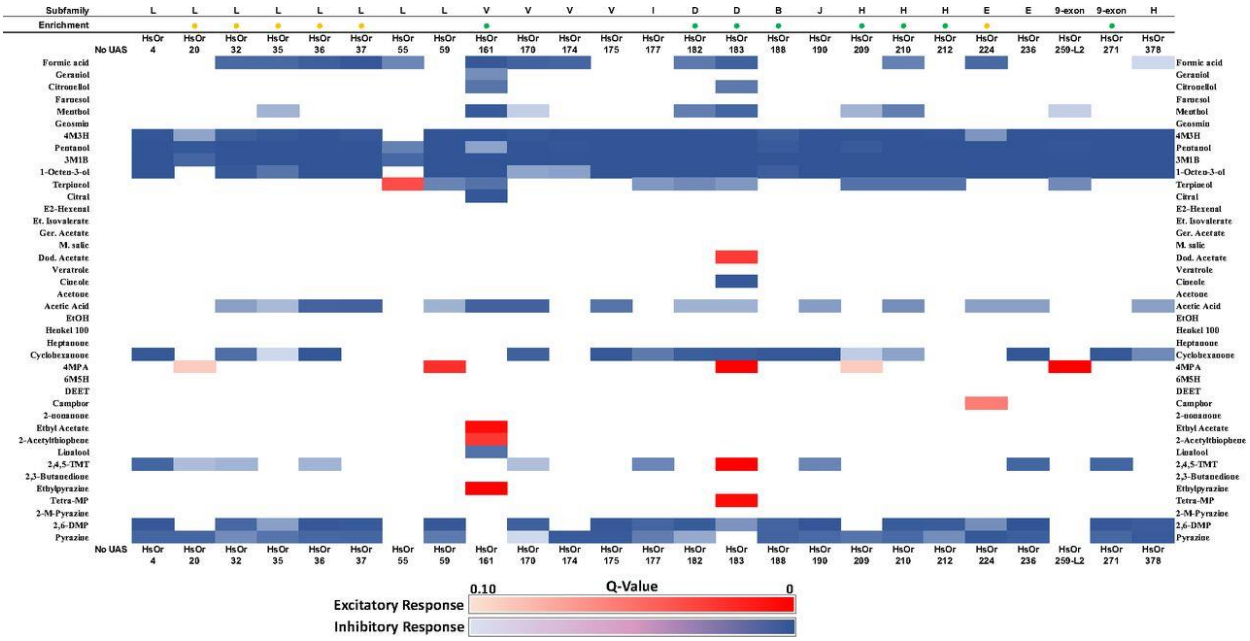

Figure A-3. Statistical analysis of EAG responses to general odorants.
Statistical analysis of EAG responses to general odorants. Analysis was conducted by using one-way ANOVA, followed by correction for multiple comparisons using a two-stage FDR method (Benjamini, Krieger, and Yekutieli 2006) with a threshold of $q < 0.10$. Significant excitatory responses are indicated in red, and inhibitory hits are indicated in blue.

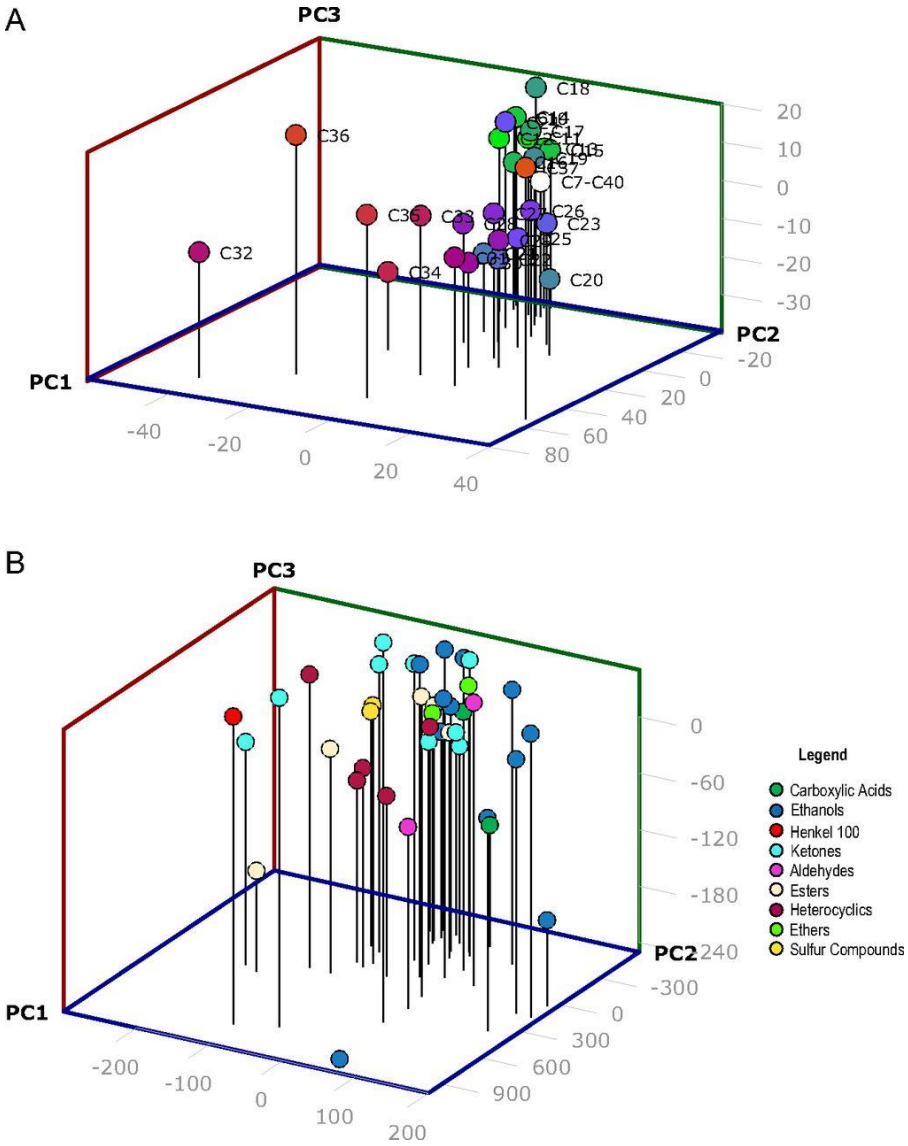

Figure A-4. PCA of variance in ligand responses.
PCA of variance in ligand responses. The first three principal components are shown for the CHC response data (*A*) and the general odorant response data (*B*). The CHC responses show a particularly clear pattern of segregation along the principal component 1 (PC1) and principal component 2 (PC2) axes, with the longer-chain CHCs generally positioned toward the front-left part of the graph and the shorter-chain CHCs positioned more toward the rear-right section of the graph. For consistency, analyses in both *A* and *B* were performed on data before background subtraction of the Orco-Gal4 response.

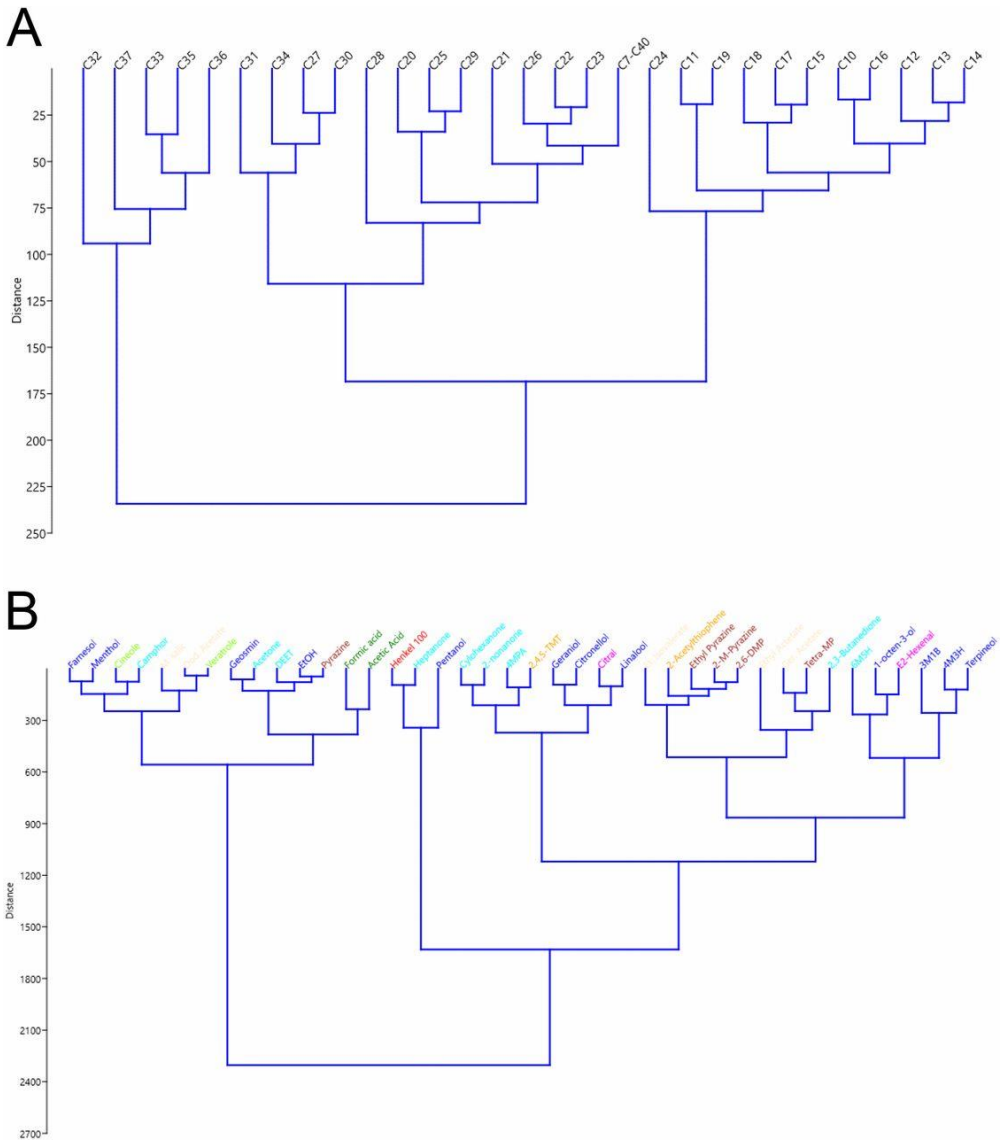

Figure A-5. Hierarchical cluster analysis of odorant responses to ligands.
Hierarchical cluster analysis of odorant responses to ligands. Analysis was performed in PAST 3, based on the diluent-adjusted data for the SSR responses to CHCs (*A*) and the EAG responses to general odorants (*B*). Distances are based on Euclidean distances between ligands.

Table A-1. Percentage of total variation accounted for by each principal component.

Principal component	Variance (SSR), %	Variance (EAG), %
1	49.824	81.965
2	9.071	5.3733
3	7.8264	4.4292
4	6.5168	2.4759
5	4.9327	1.5678
6	3.9549	1.1542
7	3.3621	0.73217
8	3.075	0.38766
9	2.3536	0.35369
10	1.6465	0.3157
11	1.4048	0.26067
12	1.1818	0.18701
13	1.0174	0.1676
14	0.74656	0.13216
15	0.65659	0.095231
16	0.51317	0.087467
17	0.42462	0.07727
18	0.36747	0.07125
19	0.27962	0.051365
20	0.18266	0.031849
21	0.14629	0.025577
22	0.10462	0.019361
23	0.070552	0.016137
24	0.066399	0.011878
25	0.009485	0.006756
26	0.003646	0.004244

Table A-2. Principle Components from the SSR and EAG recordings.

SSR								
Ligand	PC 1	PC 2	PC 3	PC 4	PC 5	PC 6	PC 7	PC 8
C10	-36.148	-9.7452	8.6812	0.084579	4.5813	0.050943	0.61066	4.8681
C11	-27.649	-2.1276	6.8916	1.4115	5.6187	3.0928	5.8783	-3.9212
C12	-26.43	-8.7609	5.9773	3.9108	-1.1706	7.0911	13.987	0.239
C13	-34.881	-1.6083	2.417	-2.6465	-0.28505	-6.6377	9.6358	11.869
C14	-33.129	-7.7921	10.006	-6.12	-3.0774	-3.5819	8.7115	-0.66851
C15	-35.66	-0.49235	1.9965	-5.1084	-0.95133	-0.32156	-4.8094	0.55085
C16	-30.121	-6.8974	-0.91728	-4.7009	12.571	0.95454	-3.0423	2.3991
C17	-29.618	-2.4458	8.2429	0.26524	1.2107	-3.8814	-10.337	1.2522
C18	-27.069	0.071953	20.654	-5.6189	2.1597	-4.3195	0.11414	-5.37
C19	-20.337	2.8986	4.4329	3.5294	15.239	-1.3831	-4.0228	-7.1247
C20	1.3383	17.143	-19.505	14.103	9.9925	4.4256	-9.4364	-4.7377
C21	-8.1875	-3.8189	-18.783	2.7358	1.1767	-13.147	-4.9265	5.4167
C22	-2.5203	2.4461	-17.714	13.537	-5.648	3.7276	2.0207	-9.7953
C23	-6.4609	12.686	-7.6234	0.67176	-2.6743	-0.3258	0.60541	-9.2202
C24	-14.216	-1.2912	14.571	-21.83	-15.537	2.8582	-17.9	-3.0671
C25	-0.66848	8.2751	-10.789	-6.2923	-5.7799	8.2509	-16.424	-5.7141
C26	-11.023	6.5516	-6.463	5.7967	-14.729	-0.53032	4.2008	-0.05126
C27	10.77	7.7781	-1.5066	-6.8896	0.56452	-13.073	6.1215	3.4763
C28	2.7868	-3.6451	-8.2114	-2.716	-15.863	30.636	9.8558	14.071
C29	17.929	12.428	-5.9656	-1.4079	1.1938	4.3993	-2.3278	-1.3995

C30	21.578	6.6853	-11.974	-19.753	4.1299	-3.6158	10.644	-7.2393
C31	37.549	10.945	-5.8964	-19.902	14.365	5.3408	-8.7115	16.975
C32	64.476	-39.617	-6.4935	-15.833	8.9095	0.78495	5.4852	-13.514
C33	33.682	0.65906	2.3304	12.126	-7.3267	-17.486	-3.7013	16.284
C34	18.104	-14.979	-19.03	9.5343	6.5752	-5.6803	5.1884	7.0393
C35	58.879	-0.61785	8.6788	-0.40883	-27.713	-10.493	-0.10521	-5.1883
C36	49.168	-22.915	23.288	26.655	6.674	9.9371	-12.154	2.5038
C37	55.17	37.065	26.634	4.3471	11.009	3.883	12.427	-2.4659
C7-C40	-27.312	1.1209	-3.9293	20.52	-5.2155	-0.95596	2.4127	-7.467
Ligand	PC 9	PC 10	PC 11	PC 12	PC 13	PC 14	PC 15	PC 16
C10	-4.0838	-7.3462	1.326	-3.6778	-2.8625	0.41146	-2.8958	2.4096
C11	-11.074	10.902	5.9535	-3.6371	6.8864	0.35494	5.4598	3.4756
C12	0.030711	-0.8998	0.54877	10.768	0.58071	2.4815	-1.3439	-7.2755
C13	1.0182	-2.0443	-0.83717	1.2694	-1.9077	-1.3916	3.5981	-0.11442
C14	6.7394	7.4961	1.0604	-7.6666	-3.5034	5.5185	-1.6013	-5.1418
C15	11.293	5.0231	-6.2616	8.3344	-7.3601	-2.3122	-1.5744	5.031
C16	0.6767	-4.4982	10.914	-2.8775	3.4348	-3.749	-0.29007	-3.227
C17	5.6249	-4.8912	0.33073	-3.9704	0.19194	1.0677	5.0714	-3.0267
C18	7.031	2.4736	0.3715	-2.2956	2.8474	-3.4584	-7.7347	4.4628
C19	-2.1053	5.9777	2.4361	6.5218	-3.5007	-3.2941	0.54741	2.6168
C20	0.7158	4.2297	3.0492	-0.85539	-4.067	-6.5397	-4.0592	-6.1323
C21	-8.529	-1.8263	7.3226	8.3547	0.89315	4.9803	2.0864	0.81565
C22	7.272	3.7414	1.2553	-1.1037	7.2134	4.2929	-1.3915	0.47303
C23	0.15797	-10.228	-0.83151	1.0899	-1.0049	6.6818	-4.1036	-1.2781

C24	-11.314	-1.2352	-4.4545	-1.4685	2.3423	-0.42945	2.3741	-2.5808
C25	0.65737	0.7797	-4.7851	-0.49305	3.5734	-3.7221	1.9606	-1.3771
C26	14.075	-0.8478	-5.3297	-1.4366	-1.8602	0.020805	7.9915	-0.11194
C27	-9.6858	-6.2805	-6.7923	6.1502	-1.1301	-4.5416	-0.29271	-1.138
C28	-4.0235	0.46343	1.86	1.4383	1.0316	-4.0048	-2.3943	1.8705
C29	0.67608	2.3573	6.0584	2.1012	-4.6549	4.4062	5.779	4.9824
C30	-9.9296	10.556	-8.9143	-2.4493	-1.4619	3.9044	-2.7387	-0.83007
C31	7.1759	-3.2977	1.4409	-2.9096	0.81559	6.5623	-3.566	1.9305
C32	8.6012	-5.6595	-0.0288	2.9248	4.3113	-3.1463	2.1675	0.64759
C33	4.5448	7.8165	-2.8935	2.1184	11.29	-1.7784	-2.5095	-0.65323
C34	-4.1855	-0.2552	-4.5114	-11.54	-6.7034	-4.3038	1.8051	0.48291
C35	-1.5742	0.38492	13.077	-1.5555	-7.2207	-1.9267	-2.9344	0.53881
C36	-5.0104	3.0642	-5.9302	1.7871	-4.2129	5.1765	-0.13062	-1.071
C37	1.3491	-4.9701	-0.14544	-1.0422	2.0105	-2.2925	3.5901	-0.93366
C7-C40	-6.1228	-10.986	-5.2887	-3.8792	4.0274	1.0311	-2.8702	5.1546
Ligand	PC 17	PC 18	PC 19	PC 20	PC 21	PC 22	PC 23	PC 24
C10	5.2746	-1.5175	-4.413	-0.06586	1.5646	-3.6049	0.54478	-1.0169
C11	-2.7603	1.7196	-1.1085	-0.48615	-1.5511	-1.9499	0.90609	-0.63456
C12	-3.976	-3.0622	-3.944	2.044	-2.1392	1.0236	0.006193	0.11249
C13	-2.5135	-0.20757	5.5279	-0.98265	1.0116	-0.95608	-0.29386	1.151
C14	2.389	-1.3332	0.49412	1.3462	2.6995	0.27055	0.34091	-0.95807

C15	4.3654	1.1282	-0.93109	1.4408	-1.7535	-0.67228	-0.23271	1.9746
C16	-0.57536	-0.41526	-0.65457	-1.5348	0.5615	0.11942	-2.2998	2.9025
C17	-0.096446	8.3839	0.54447	2.2561	-1.5798	1.1469	2.258	0.32919
C18	-2.8439	-3.3283	1.8502	-3.0864	-2.3979	1.7681	1.5949	-0.67439
C19	-1.8241	2.9706	0.79719	0.41951	3.6745	1.4727	-2.2014	-1.7326
C20	-0.90864	-0.58827	1.7277	1.4143	-1.1775	-0.99404	0.74372	-1.6257
C21	4.3037	-4.1801	4.3532	-0.27662	-0.61895	0.51661	1.5009	-0.25167
C22	4.5573	1.3286	-2.0603	-2.7098	2.3461	2.3599	0.59876	1.1812
C23	-1.0901	4.3078	0.92464	-3.6932	-2.5251	-2.1086	-1.7222	-0.78906
C24	3.6089	-1.9643	0.20533	-0.08057	-1.0001	1.9618	-1.8349	-1.0222
C25	-3.6286	-3.6619	-1.1534	-0.14735	1.7997	-2.16	1.4523	1.5345
C26	-2.5877	-2.6867	0.7135	-1.8278	0.75136	-0.79517	-0.80056	-1.5041
C27	-1.5188	2.5612	-3.705	-2.5511	2.4908	1.1799	1.8587	-0.38085
C28	1.8272	3.1537	2.1283	-0.30316	0.1955	0.51425	0.2593	-0.59457
C29	-0.89132	-1.281	-4.0477	0.63263	-1.5663	0.78243	-0.40324	-0.1853
C30	-0.32279	1.3236	1.713	1.116	-0.26369	-0.85809	-0.29091	1.5073
C31	-3.9912	-0.65837	-0.47603	1.2699	1.0883	0.79059	0.2297	-0.58936
C32	1.3001	0.26498	1.2041	1.1593	-0.19356	-0.80446	0.10415	-0.96725

C33	0.94025	1.5721	-1.6097	1.1666	-0.89784	-1.2475	-1.6037	-0.60724
C34	0.36792	-1.4283	-2.0208	-1.1863	-2.5011	2.0615	-0.67006	0.13747
C35	-2.4045	0.96843	-0.22425	0.96116	0.74562	-0.40648	0.19465	0.8608
C36	-1.3046	-0.23329	1.5271	-2.1592	0.658	-0.37129	0.275	0.84351
C37	5.0496	-1.7965	1.2025	0.62599	-0.31856	0.13409	0.071912	0.64536
C7-C40	-2.5634	-1.3401	1.4352	5.2385	0.89722	-0.82625	-0.58666	0.35374
Ligand	PC 25	PC 26						
C10	-0.1914	-0.50118						
C11	-0.25891	0.45981						
C12	0.012394	-0.23717						
C13	0.8096	-0.05902						
C14	0.57402	0.47993						
C15	-0.18834	0.43884						
C16	-0.6213	0.14447						
C17	-0.10289	-0.37758						
C18	-0.003836	-0.24034						
C19	0.24217	-0.38531						
C20	-0.24416	0.2064						
C21	-0.18431	-0.07278						
C22	0.040849	-0.10741						
C23	0.50534	0.1583						
C24	-0.042119	0.095635						

C25	0.84309	-0.16351						
C26	-1.1526	-0.10667						
C27	-0.20896	0.45986						
C28	0.032308	-0.05028						
C29	0.61343	-0.03541						
C30	-0.57737	-0.46238						
C31	-0.33152	0.16645						
C32	0.14979	0.11317						
C33	0.27011	-0.09345						
C34	0.41269	-0.08088						
C35	-0.1615	-0.05179						
C36	-0.22215	0.09533						
C37	0.11125	-0.03057						
C7-C40	-0.12567	0.23749						
EAGs								
Ligand	PC 1	PC 2	PC 3	PC 4	PC 5	PC 6	PC 7	PC 8
Formic acid	-582.87	-37.638	-34.486	-104.14	-42.687	18.382	33.102	-9.4358
Geraniol	-9.7892	40.172	98.954	-98.603	-38.411	36.038	-5.1149	-8.8162
Citronellol	-109.04	48.531	82.21	-81.784	-58.753	18.081	-2.2083	26.921
Farnesol	-244.34	6.2531	10.603	-25.215	-33.045	27.159	5.2751	-8.3054
Menthol	-254.46	88.618	41.707	-7.4595	-42.193	13.493	-4.2165	18.435
Geosmin	-344.9	-25.161	-31.171	27.775	-17.035	38.148	23.624	3.8339
4M3H	157.05	168.11	20.075	-3.9388	-13.678	46.897	9.7236	25.166
Pentanol	855.81	52.548	-253.05	-41.158	-20.471	89.07	-2.0606	-41.303

3M1B	36.334	188.94	-158.83	-32.282	5.2829	36.325	-66.076	37.855
1-octen-3-ol	360.23	165.68	-23.649	21.314	31.176	0.31604	63.291	37.696
Terpineol	163.4	189.48	51.75	87.073	51.66	-42.343	37.365	-4.484
Citral	0.78974	81.735	50.763	-0.76965	89.585	-6.6685	13.584	-18.471
E2-Hexenal	321.36	49.714	-56.914	48.675	2.8073	-59.406	-0.89712	-53.917
Et. Isovalerate	167.43	-84.785	-11.695	20.069	-69.668	-10.879	19.201	-4.8979
Ger. Acetate	196.22	45.521	69.242	-27.044	78.161	-22.753	19.818	2.8541
M. salic	-184.89	15.119	-10.03	95.67	19.145	-21.389	21.67	-14.425
Dod. Acetate	-307.87	-28.182	-0.505	26.157	55.39	19.056	2.9521	-4.1385
Veratrole	-282.19	-25.535	-5.5973	43.963	35.918	6.6887	-6.7944	-16.073
Cineole	-277.73	24.618	32.777	36.159	-42.731	-10.901	36.849	-24.276
Acetone	-334.79	2.0833	-40.954	28.115	-12.668	-19.513	14.028	-2.5839
Acetic Acid	-360.36	38.662	-120.69	-193.43	33.277	-145.87	-13.533	-5.2798
EtOH	-412.91	-34.209	-4.08	22.91	-0.02564	9.364	3.8773	-13.32
Henkel 100	787.75	-105.74	77.369	-4.9517	-8.7873	-14.958	-8.0508	3.6555
Heptanone	722.88	-54.27	100.12	-27.616	-37.801	-35.298	-14.717	-40.681
Cylohexanone	-97.516	2.3517	-13.862	10.504	-39.456	-32.469	-38.12	-0.90098
4MPA	-103.88	-66.711	55.905	-49.946	48.568	15.655	-27.177	-3.6772
6M5H	480.45	44.653	153.27	-6.5747	-21.221	-35.236	-33.671	8.7338
DEET	-392.08	-13.304	-34.183	60.848	29.53	-7.4362	14.951	-14.251
Camphor	-240.03	32.776	64.937	51.859	-45.168	-6.9758	-18.024	27.192
2-nonanone	-103.25	-18.636	65.003	2.6973	-37.474	10.645	-29.996	4.3244
Ethyl Actetate	294.34	-162.88	-142.87	73.431	-16.245	-82.435	-7.5506	70.8

2-Acetylthiophene	5.3449	-55.603	24.331	34.317	-69.166	1.4486	-25.91	-4.8381
Linalool	28.376	13.175	81.878	-37.326	2.3493	13.14	-10.478	-2.485
2,4,5-TMT	-137.3	-84.1	-0.28502	20.213	111.72	42.662	-89.423	-11.738
2,3-Butanedione	258.85	-183.87	-12.791	-111.8	52.556	33.165	92.674	17.638
Ethyl Pyrazine	53.227	-60.564	-38.592	62.395	-60.205	11.082	31.52	-1.6054
Tetra-MP	160.64	-114.23	61.706	9.5534	89.304	44.637	-3.8392	31.271
2-M-Pyrazine	22.087	-74.088	-57.042	14.215	2.0601	1.2382	-15.415	10.919
2,6-DMP	88.032	-22.117	-58.273	34.821	-1.8407	-0.09579	-17.167	-6.0744
Pyrazine	-380.4	-47.098	-33.05	21.303	-9.7642	21.937	-3.0667	-11.316
Ligand	PC 9	PC 10	PC 11	PC 12	PC 13	PC 14	PC 15	PC 16
Formic acid	-11.114	8.7396	7.5373	-14.073	10.178	20.934	-12.398	19.206
Geraniol	-39.123	42.613	-13.607	21.369	34.566	2.6742	-5.9307	-13.353
Citronellol	-19.42	20.251	14.051	15.337	-9.0745	-8.8452	2.8588	-9.8449
Farnesol	-30.57	-20.038	7.3575	-15.736	-19.1	10.09	-0.82451	-11.85
Menthol	-35.396	-26.194	16.535	-4.387	-12.066	5.7476	15.586	-2.9161
Geosmin	-9.07	-8.0427	7.0979	-0.0142	-4.0973	-1.0162	-17.047	4.4316
4M3H	4.3504	0.39142	13.105	17.923	-6.7877	-5.8952	19.545	17.888
Pentanol	4.2151	-8.0592	13.183	28.255	-11.015	6.0175	-10.22	6.439
3M1B	-3.0287	7.4006	-41.49	-39.884	9.3766	-10.611	-5.8984	-2.0045
1-octen-3-ol	25.752	-14.277	-16.703	-6.9756	22.157	3.1484	1.7594	-12.693
Terpineol	-29.297	-17.314	32.333	-7.5015	-4.5569	24.119	-7.9742	0.61058
Citral	8.0274	19.315	16.584	2.1313	7.3778	-18.039	-9.1797	16.128
E2-Hexenal	-25.905	50.326	11.021	-38.727	-16.972	-6.419	8.6364	-10.768
Et. Isovalerate	22.958	-10.407	4.2992	-25.536	24.451	-25.274	-9.5084	-2.6331

Ger. Acetate	-2.5992	29.125	-34.435	9.5327	-19.94	3.4886	-1.7806	5.257
M. salic	-18.588	4.6049	-3.5413	13.387	16.595	-19.979	14.151	-4.4264
Dod. Acetate	-12.505	-11.6	-8.9621	16.893	1.2994	4.2677	1.5685	8.2254
Veratrole	-14.096	-18.397	-20.498	9.0134	7.1195	-3.7687	13.726	-13.015
Cineole	24.891	6.3326	-26.56	12.362	-11.077	-7.6659	-1.4493	8.1987
Acetone	20.737	-6.526	-27.459	-1.5498	-14.829	-0.82423	-15.299	-7.7548
Acetic Acid	26.054	-6.8848	14.581	14.049	9.0942	10.495	5.4491	-4.9614
EtOH	0.70106	-3.6252	-18.36	-8.4888	-2.593	5.3757	-3.3126	12.802
Henkel 100	-8.4809	11.245	-28.856	2.3783	23.42	18.238	19.028	15.656
Heptanone	-14.911	-49.135	-23.639	-3.4683	-3.3638	5.3314	-12.218	4.7701
Cylohexanone	-6.4486	-20.833	-21.177	33.706	-17.757	-19.072	4.4189	-11.957
4MPA	1.3401	19.101	14.478	-11.121	-4.4032	-29.794	-7.7609	13.63
6M5H	24.654	-17.654	15.672	-4.9495	1.2853	-3.4239	-13.297	-8.2295
DEET	2.7145	3.0939	-5.3382	8.3614	10.154	6.7592	-7.6906	6.2496
Camphor	9.7483	-5.9595	15.461	4.7909	-2.4713	-9.3938	-12.616	4.6525
2-nonanone	21.306	-2.5767	7.1726	-22.975	5.9127	15.273	22.14	21.188
Ethyl Actetate	-44.97	5.375	3.8837	9.7137	-1.2003	0.080471	-12.179	14.913
2-Acetylthiophene	1.4843	40.857	1.8042	1.6412	-20.224	4.9653	-1.7169	-5.7273
Linalool	34.397	-20.22	7.8259	-3.2231	-10.716	-2.4936	-5.6243	0.70008
2,4,5-TMT	-20.199	-22.431	24.256	-1.2219	21.535	3.1556	-3.1651	-9.9207
2,3-Butanedione	-18.857	-21.644	6.0916	-18.599	-11.422	-23.123	12.651	-9.0645
Ethyl Pyrazine	24.565	18.409	29.628	6.9787	30.882	12.673	-2.9923	-18.777
Tetra-MP	35.332	26.953	-5.6424	5.6096	-17.294	21.711	-11.386	-15.107
2-M-Pyrazine	24.503	10.154	11.789	-7.7647	-25.325	17.026	17.471	-7.4627

2,6-DMP	32.845	2.3009	24.052	11.664	3.5037	-17.525	18.768	6.6966
Pyrazine	14.001	-14.77	-23.533	-8.9001	7.3777	11.593	13.713	-5.1766
Ligand	PC 17	PC 18	PC 19	PC 20	PC 21	PC 22	PC 23	PC 24
Formic acid	5.1653	29.723	5.4936	6.2054	3.481	-2.9429	-2.8051	-1.7291
Geraniol	-14.831	-5.6955	6.284	1.1369	0.44948	10.584	-3.8824	4.3075
Citronellol	0.15463	-2.6162	-6.4491	2.9204	-1.2448	-8.6242	-5.0594	-2.1617
Farnesol	-8.8249	-10.066	-2.9314	1.6792	16.284	-2.1979	3.1481	-5.715
Menthol	-2.8657	-16.991	-5.6643	-0.54642	-9.1137	-1.049	0.19399	-1.8688
Geosmin	-1.9331	-4.1694	-14.753	-1.1824	-10.205	-5.5166	10.3	3.7073
4M3H	18.675	7.0809	-3.6419	-4.5625	6.1578	8.6314	8.7145	0.72692
Pentanol	-3.0863	-7.5195	-2.1474	0.64286	-0.66606	2.6395	-2.8883	-0.43771
3M1B	9.7208	-3.3577	7.8685	3.7919	-0.45483	-6.5506	-0.07454	5.0584
1-octen-3-ol	-11.54	17.767	-4.4183	-6.2024	-0.44663	-0.63772	1.444	-8.7559
Terpineol	6.1196	-3.0579	16.069	4.3919	-5.5624	-0.12125	-6.4217	5.0885
Citral	-4.6121	-0.91753	5.7402	-0.30804	-8.192	0.24302	-2.7857	-7.4494
E2-Hexenal	-6.8246	4.883	-11.35	5.7038	-2.0335	4.2243	5.3586	-2.6416
Et. Isovalerate	-1.9005	-18.588	5.3722	1.4983	7.3848	1.9001	0.41495	-3.2784
Ger. Acetate	-16.003	-2.9138	-2.1601	-15.552	9.0267	-7.5101	-0.83379	1.8577
M. salic	10.454	-0.70614	7.0738	2.9673	13.195	-0.14927	-0.36124	2.6958
Dod. Acetate	0.16085	-1.2271	-12.786	7.851	7.9809	-3.1042	-0.08723	-5.7727
Veratrole	6.2926	3.2132	-7.1061	1.7433	-5.1961	-8.6581	-3.3228	4.7666
Cineole	0.45909	-2.1154	-3.5574	6.2164	-4.2031	-1.0726	-6.3285	3.4024
Acetone	3.7194	3.2713	-11.082	-6.5635	-1.2803	11.9	-5.4877	2.5325

Acetic Acid	8.433	-9.8692	-1.1115	0.39241	0.16876	-0.3252	4.3761	-0.26635
EtOH	9.1795	-11.519	2.4274	-3.9339	-2.9486	1.4225	-3.1396	-4.8594
Henkel 100	18.322	-7.6502	-10.801	4.6235	-3.7929	-0.37132	0.94081	-1.4366
Heptanone	3.9008	3.4332	12.267	-5.4386	1.8447	-6.5533	2.0466	-1.0915
Cylohexanone	-9.1512	9.9171	16.171	-1.3506	-9.798	2.9494	5.7913	-4.6514
4MPA	7.5169	-6.0636	-4.1558	-9.042	-5.1452	-2.0251	-2.432	-1.6586
6M5H	6.7248	9.2351	-17.618	7.8537	0.72458	4.6276	-1.6979	3.3847
DEET	-1.137	-7.3149	3.4038	-1.3833	3.5269	3.4762	11.559	7.4894
Camphor	10.604	-6.212	7.801	-0.47832	4.6209	4.0501	-1.1838	-3.6087
2-nonanone	-27.627	-2.6788	7.8181	-5.6987	-4.1689	3.0158	3.6249	3.0342
Ethyl Actetate	-15.01	4.0564	-4.7135	1.715	0.58479	2.0659	-3.229	0.86607
2-Acetylthiophene	15.229	21.529	7.9752	-3.8167	-0.16513	-3.1494	4.9604	-0.18266
Linalool	-16.629	5.4497	-7.4596	3.1901	5.3032	-1.5066	-0.06516	7.9127
2,4,5-TMT	0.2239	13.454	-3.4253	-8.0212	1.7026	3.6742	-0.73822	-0.65351
2,3-Butanedione	6.2007	6.0971	7.7493	0.046461	-3.9399	4.4804	1.1557	5.212
Ethyl Pyrazine	3.8772	-3.3782	-1.5617	-14.028	-4.3521	-9.0471	1.0836	0.55177
Tetra-MP	1.5878	-11.723	11.761	16.137	-1.8698	0.087127	6.0175	-2.8581
2-M-Pyrazine	8.3281	-5.0575	4.1339	-11.161	4.739	3.0369	-10.916	1.0744
2,6-DMP	-14	7.4347	5.5815	12.382	2.9517	-8.2245	-3.6918	2.0392
Pyrazine	-5.074	4.8617	-2.0978	6.1804	-5.3477	6.3276	-3.6982	-4.6312
Ligand	PC 25	PC 26						
Formic acid	1.3592	2.3553						
Geraniol	-2.0628	0.56179						
Citronellol	0.93189	-1.2135						
Farnesol	-1.8579	-5.7507						
Menthol	-1.2188	4.112						

Geosmin	1.8346	-0.53041						
4M3H	2.4116	1.24						
Pentanol	0.88282	-0.53804						
3M1B	-0.038033	-0.57589						
1-octen-3-ol	-5.2637	-1.3992						
Terpineol	1.8856	0.43467						
Citral	2.3155	-1.0862						
E2-Hexenal	-1.8558	1.9386						
Et. Isovalerate	5.8976	2.8937						
Ger. Acetate	5.6224	1.1747						
M. salic	2.8347	-0.34006						
Dod. Acetate	-1.1608	3.5186						
Veratrole	0.55653	1.3555						
Cineole	-5.1122	-5.962						
Acetone	-1.8018	3.6092						
Acetic Acid	-0.18046	-0.62648						
EtOH	4.9823	-4.3225						
Henkel 100	-1.4732	-0.09297						
Heptanone	-4.0477	2.1305						
Cylohexanone	3.0072	-0.13012						
4MPA	-4.8619	1.0819						
6M5H	4.9456	-4.5118						
DEET	-4.4467	-1.5134						
Camphor	-6.1586	1.8913						
2-nonanone	0.22927	-2.0728						
Ethyl Actetate	0.46312	-0.80427						
2-Acetylthiophene	0.97072	-0.72686						
Linalool	0.99673	4.1232						
2,4,5-TMT	-0.50208	-2.2525						
2,3-Butanedione	-0.37242	-1.7031						
Ethyl Pyrazine	0.9364	0.46829						

Tetra-MP	-0.09497	1.3537						
2-M-Pyrazine	-1.5221	0.16439						
2,6-DMP	-3.1598	0.32451						
Pyrazine	4.1277	1.4208						

Table A-3. List of odorants used in this study, their abbreviations, and Chemical Abstracts Service (CAS) registry numbers

Abbreviation	Full name	CAS no.
Formic acid	Formic acid	64-18-6
Geraniol	Geraniol	106-24-1
Citronellol	Citronellol	106-22-9
Farnesol	Farnesol	4602-84-0
Menthol	Menthol	89-78-1
Geosmin	Geosmin	16423-19-1
4M3H	4-Methyl-3-heptanol	14979-39-6
Pentanol	Pentanol	71-41-0
3M1B	3-Methyl-1-butanol	123-51-3
1-Octen-3-ol	1-Octen-3-ol	3391-86-4
Terpineol	Terpineol	98-55-5
Citral	Citral	5392-40-5
E2-Hexenal	*trans*-2-Hexenal	6728-26-3
Et. isovalerate	Ethyl isovalerate	108-64-5
Ger. acetate	Geranyl acetate	105-87-3
M. salic	Methyl salicylate	119-36-8
Dod. acetate	Dodecyl acetate	112-66-3
Veratrole	Veratrole	91-16-7
Cineole	Cineole	470-82-6
Acetone	Acetone	67-64-1
Acetic acid	Acetic acid	64-19-7.
EtOH	Ethanol	64-17-5
Henkel 100	Henkel 100	Not available
Heptanone	2-Heptanone	110-43-0
Cyclohexanone	Cyclohexanone	108-94-1
4MPA	4-Methoxyphenylacetone	122-84-9
6M5H	6-Methyl-5-hepten-2-one	110-93-0
DEET	DEET	134-62-3
Camphor	Camphor	76-22-2
2-Nonanone	2-Nonanone	821-55-6
Ethyl acetate	Ethyl acetate	141-78-6
2-Acetylthiophene	2-Acetylthiophene	88-15-3
Linalool	Linalool	78-70-6
2,4,5-TMT	2,4,5-Trimethylthiazole	13623-11-5

Abbreviation	Full name	CAS no.
2,3-Butanedione	2,3-Butanedione	431-03-8
Ethylpyrazine	Ethylpyrazine	13925-00-3
Tetra-MP	Tetramethylpyrazine	1124-11-4
2-M-Pyrazine	2-Methylpyrazine	109-08-0
2,6-DMP	2,6-Dimethylpyrazine	108-50-9
Pyrazine	Pyrazine	290-37-9
C5	Pentane	109-66-0
C10	*n*-Decane	124-18-5
C11	*n*-Undecane	1120-21-4
C12	*n*-Dodecane	112-40-3
C13	*n*-Tridecane	629-50-5
C14	*n*-Tetradecane	629-59-4
C15	*n*-Pentadecane	629-62-9
C16	*n*-Hexadecane	544-76-3
C17	*n*-Heptadecane	629-78-7
C18	*n*-Octadecane	593-45-3
C19	*n*-Nonadecane	629-92-5
C20	*n*-Icosane	112-95-8
C21	*n*-Heneicosane	629-94-7
C22	*n*-Docosane	629-97-0
C23	*n*-Tricosane	638-67-5
C24	*n*-Tetracosane	646-31-1
C25	*n*-Pentacosane	629-99-2
C26	*n*-Hexacosane	630-01-3
C27	*n*-Heptacosane	593-49-7
C28	*n*-Octacosane	630-02-4
C29	*n*-Nonacosane	630-03-5
C30	*n*-Triacontane	638-68-6
C31	*n*-Hentriacontane	630-04-6
C32	*n*-Dotriacontane	544-85-4
C33	*n*-Tritriacontane	630-05-7
C34	*n*-Tetratriacontane	14167-59-0
C35	*n*-Pentatriacontane	630-07-9
C36	*n*-Hexatriacontane	630-06-8
C37	*n*-Heptatriacontane	7194-84-5

Abbreviation	Full name	CAS no.
C7-C40	C7 to C40 Mixture	Not available
2-MeC28	2-Methyloctacosane	1560-98-1
9-C29:1	(Z)-9-Nonacosene	36258-10-3
3-MeC29	3-Methylnonacosane	14167-67-0
13-MeC29	13-Methylnonacosane	7371-98-4
15-MeC29	15-Methylnonacosane	65820-60-2
2-MeC30	2-Methyltriacontane	1560-72-1
9-C31:1	(Z)-9-Hentriacontene	56987-72-5
5-MeC31	5-Methylhentriacontane	71502-24-4
13-MeC31	13-Methylhentriacontane	33116-06-2
15-MeC31	15-Methylhentriacontane	Not available
13,23-DimeC37	13,23-Dimethylheptatriacontane	Not available

Appendix B. Chapter II supplementary materials and methods[‡‡]

Odorant Receptor Cloning.

The cloning of the *HsOr* genes followed standard protocols. Full-length *Or* coding sequences were PCR-amplified from adult worker or male antennal cDNA and cloned by using the Gateway system (Life Technologies), or commercially synthesized (Genscript). For transgenic expression of *HsOr* genes in flies, we have subcloned our genes into a p*UAS* plasmid that allows for the insertion of our transgenes into a preexisting insertion site in the *Drosophila* genome, using the well-established phiC31 integrase recombination system (Groth et al. 2004).

***Drosophila* Genetics.**

For SSR and EAG experiments, experimental *D. melanogaster* genotypes were either w^{1118}*;* w^{+}, *UAS-HsOr;* w^{+}, *Orco-GAL4* or w^{1118}; *+ ;* w^{+}, *UAS-HsOr/*w^{+}, *Orco-GAL4*. Control flies were w^{1118}*; +;* w^{+}, *Orco-GAL4*. Embryo injections to generate *UAS-HsOr* lines was outsourced to a commercial injection service (Rainbow Transgenic Flies).

Electrophysiology.

Flies were tested 2–10 d after eclosion for both single-sensillum and whole antennal EAG recordings, with an $n = 4–8$ per *UAS-HsOrX* line. For SSRs, odorants and volatilized hydrocarbons were puffed by using a Syntech CS-05 Stimulus Flow Controller (Ockenfels SYNTECH). The ab2 sensillum-type was used for all recordings and was confirmed by using a "diagnostic odorant" panel consisting of paraffin oil (negative control), ethyl acetate, geranyl acetate, 1-octen-3-ol, 2-heptanone, and (*E*)-2-hexenal. Responses were measured from the ab2A neuron, which can be separated from ab2B neurons based on spike amplitude (de Bruyne, Foster, and Carlson 2001). Each odorant was diluted to 0.01 M in paraffin oil, and 20 μL of the resulting solution was loaded into each delivery cartridge. For hydrocarbons, each compound was dissolved in pentane and 20 nmol of the compound was applied to each delivery cartridge, with the pentane solvent allowed to evaporate. The cartridges were then heated for 1 s with a handheld butane torch (BernzOmatic,

[‡‡] This chapter was published in 2017 in the *Proceedings of the National Academy of Sciences*, *114*(32). Jesse. D. Slone was first author. Gregory M. Pask, Stephen T. Ferguson, Jocelyn G. Millar, Shelley L. Berger, Danny Reinberg, Jürgen Liebig, and Anandasankar Ray were co-authors. L.J. Zwiebel was senior author. I contributed Figure II-4, Figure A-1, Figure A-2, and Figure A-3 in addition to contributing to the writing and revision of the manuscript.

Worthington Industries), then air was puffed through the heated cartridge into an airstream and over the fly antenna for a 500-ms duration of 3 mL of humidified air. There was an ~300-ms delay between the initiation of the odorant puff and the odorant actually reaching the antenna.

Odorant responses were calculated by counting spike frequencies (from the ab2A neuron) in response to odorant stimulation, although there is mounting evidence that changes in spike amplitude and local field potentials can also carry olfactory information in certain cases (Martin and Alcorta 2016). To maintain uniformity in analyses, the response was calculated by manually counting spikes in a 200-ms period between 400 and 600 ms after initiation of the stimulus, which (because of the 300-ms delay) lies within the stimulus window for the 500-ms puff. The response during the 200-ms period was multiplied by 5 to convert the response rate to spikes per s, and the prestimulus spiking rate during the 1,000 ms directly preceding the odorant puff was subtracted from the response rate to obtain the response relative to baseline.

EAG recordings were performed according to a protocol previously described by another group (Ueira-Vieira et al. 2014), with odorants diluted 1:100 by volume in paraffin oil. The EAG software (EAG2000, Ockenfels SYNTECH) initially normalizes the data to the paraffin oil control to account for day-to-day/prep-to-prep variation, responses to the solvent, and decay in the sensitivity of the antennae over the duration of the assay. We then manually normalized those responses to the Orco-GAL4 control.

Principal Component and Clustering Analysis of Receptor Responses.

PCA and hierarchical cluster analysis were performed in PAST 3 (Hammer, Harper, and Ryan 2001), based on the vehicle-adjusted data for each group of recordings. PCA was performed by using the variance-covariance matrix, and cluster analysis was performed by using Ward's method to calculate Euclidean distance.

To better understand the variance in our odorant response profiles, we carried out a PCA of all of the *HsOr*-mediated responses to the different classes of ligands tested. Overall, for the SSR data with just the volatilized hydrocarbons, more than 66% of the variation in hydrocarbon responses is explained by just three components; of that, nearly 50% is explained by just the first principal component (Table A-1). The first three components were even more critical for the EAG data with the non-CHC odorants, with just over 92% of the variation explained by the top three components and almost 82% explained by the first component alone (Table A-1). Interestingly,

both the PCA analysis (Figure A-4) and an additional hierarchical cluster analysis (Figure A-5) of the CHC data shows segregation of the hydrocarbons based on chain length, perhaps reflecting (in part) the overall lack of response to shorter chain hydrocarbons among the receptors tested. The non-CHC odorants, by contrast, show tight grouping of the strongest endogenous activators of the antenna (pentanol, heptanone, and Henkel 100) in the hierarchical clustering analysis (Figure A-5), and both analyses revealed distinct clusters for the carboxylic acids, sulfur compounds, and four of the five heterocyclics/pyrazines (Figure A-4 and Figure A-5). Similar to previous results in dipterans (Hallem and Carlson 2006), however, we observed widespread intermingling of chemical classes by using both methods, consistent with the idea that chemical class is only one variable in the overall set of characteristics that determines the sensitivity of odorant receptors.

Statistical Analysis

Statistical significance of HsOR-mediated ligand responses compared with no-UAS controls was calculated in GraphPad Prism (GraphPad Software) by using a parametric one-way ANOVA with correction for multiple comparisons using the two-stage step-up method of Benjamini, Krieger, and Yuketieli (Benjamini, Krieger, and Yekutieli 2006) at a 0.10 FDR.

Appendix C. Chapter III supplementary information[§§]

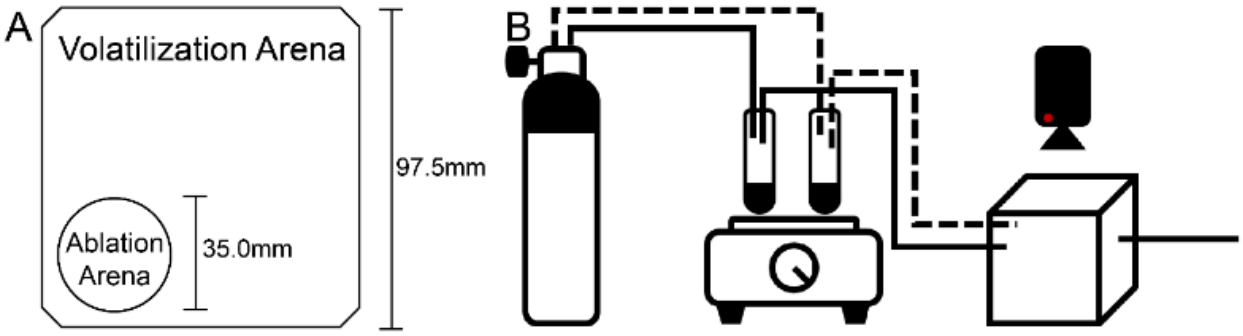

Figure C-1. Comparison of the aggression bioassay arenas (A) and a schematic of the volatilization bioassay (B).
Comparison of the aggression bioassay arenas (A) and a schematic of the volatilization bioassay (B).

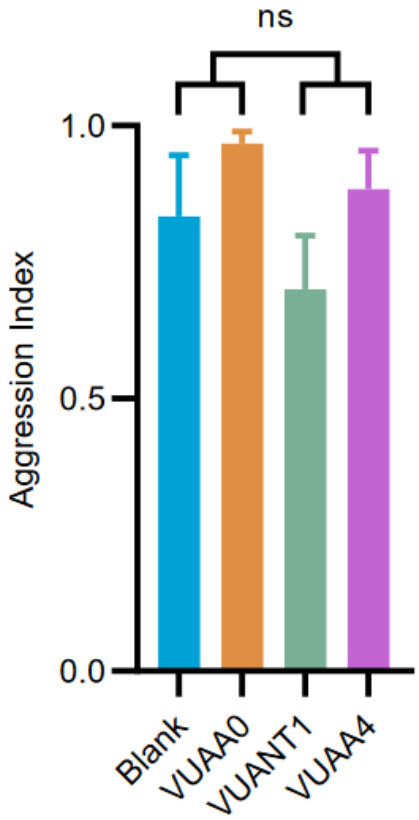

Figure C-2. Aggression in a non-social context.
Aggression (biting and wide opening of the mandibles) of individual ants in response to a mechanical stimulus from a Von Frey filament. There is no significant difference in aggression between ants exposed to either heated-air alone (Blank), VUAA0, VUANT1,or VUAA4 (Kruskal-Wallis test, Biological Replicates: Blank=11, VUAA0=10, VUANT1=10, VUAA4=10). Bars display mean. Error bars display S.E.M.

[§§] This chapter was published in 2020 in the *Journal of Experimental Biology*, *223*(2), with myself as first author. Kyu Young Park, Alexandra Ruff, and Isaac Bakis were co-authors. L.J. Zwiebel was senior author.

Table C-1. Summary of statistical test results.

Assay (N)	Dependent Variable		Result	P value	Significant?
Ablation Aggression Bioassays (6-10)	Aggression Index	Two-Way ANOVA with Holm-Sidak's Multiple Comparisons Test (with Multiplicity Adjusted P-Values)			
		Interaction	F (2, 44) = 5.331	0.0085	**
		Row Factor (Treatment)	F (2, 44) = 4.256	0.0204	*
		Column Factor (nNM/NM)	F (1, 44) = 28.92	<0.0001	****
		nNMs vs. NMs			
		Sham nNMs vs. Sham NMs	t=4.404	0.0001	***
		Unilateral nNMs vs. Unilateral NMs	t=5.438	<0.0001	****
		Bilateral nNMs vs. Bilateral NMs	t=0.2647	0.7925	ns
		Antennal Treatment			
		Sham nNMs vs. Unilateral nNMs	t=1.048	0.3005	ns
		Sham nNMs vs. Bilateral nNMs	t=3.384	0.003	**
		Sham NMs vs. Unilateral NMs	t=0.01294	0.9897	ns
		Sham NMs vs. Bilateral NMs	t=0.2336	0.9663	ns
Ablation Mobility Control Bioassay (24-29)	Distance Traveled (cm)	Kruskal-Wallis Test with Dunn's Multiple Comparisons Test (with Multiplicity Adjusted P-Values)			
		Kruskal-Wallis Statistic	H=7.246772625	0.0267	*
		Sham vs. Unilateral	mean rank diff.=-7.931034483	0.6143	ns
		Sham vs. Bilateral	mean rank diff.=9.760057471	0.4125	ns

		Unilateral vs. Bilateral	mean rank diff.=17.6910 9195	0.021 3	*
	Time Spent Moving (%)	Kruskal-Wallis Test with Dunn's Multiple Comparisons Test (with Multiplicity Adjusted P-Values)			
		Kruskal-Wallis Statistic	H=7.2653347 31	0.026 4	*
		Sham vs. Unilateral	mean rank diff.=-5.310344828	>0.99 99	ns
		Sham vs. Bilateral	mean rank diff.=12.1307 4713	0.194 7	ns
		Unilateral vs. Bilateral	mean rank diff.=17.4410 9195	0.023 9	*
	Rotational Frequency (Count)	Kruskal-Wallis Test with Dunn's Multiple Comparisons Test (with Multiplicity Adjusted P-Values)			
		Kruskal-Wallis Statistic	H=9.5535860 88	0.008 4	**
		Sham vs. Unilateral	mean rank diff.=-9.310344828	0.390 1	ns
		Sham vs. Bilateral	mean rank diff.=10.6609 1954	0.297	ns
		Unilateral vs. Bilateral	mean rank diff.=19.9712 6437	0.006	**
Electroantennography (5-6)	Solvent (Hexane) Normalized Response to Decane	Linear Regression (Is slope significantly non-zero?)			
		Blank (Y=20.37*X)	F (1, 24) = 11.39	0.002 5	**
		VUAA0 (Y=22.74*X)	F (1, 24) = 25.31	<0.00 01	****
		VUANT1 (Y=0.3758*X)	F (1, 29) = 0.02389	0.878 2	ns
		VUAA4 (Y=8.396*X)	F (1,24) = 0.0320	0.032	*
	Solvent (Paraffin Oil) Normalized	Linear Regression (Is slope significantly non-zero?)			
		Blank (Y=27.36*X)	F (1, 23) = 22.58	<0.00 01	****

	Response to 4-methyl-3-heptenol	VUAA0 (Y=30.40*X)	F (1, 23) = 42.11	<0.0001	****
		VUANT1 (Y=7.267*X)	F (1, 19) = 0.5098	0.4839	ns
		VUAA4 (Y=55.53*X)	F (1,19) = 11.65	0.0029	**
Volatile Orco Modulator Aggression Bioassay (10-12)	Aggression Index	Two-Way ANOVA with Holm-Sidak's Multiple Comparisons Test (with Multiplicity Adjusted P-Values)			
		Interaction	F (3, 79) = 3.773	0.0138	*
		Row Factor (Treatment)	F (3, 79) = 3.462	0.0202	*
		Column Factor (nNM/NM)	F (1, 79) = 14.34	0.0003	***
		nNMs vs. NMs			
		Blank nNMs vs. Blank NMs	t=3.980	0.0006	***
		VUAA0 nNMs vs. VUAA0 NMs	t=3.020	0.0102	*
		VUANT1 nNMs vs. VUANT1 NMs	t=0.7084	0.7304	ns
		VUAA4 nNMs vs. VUAA4 NMs	t=0.1570	0.8756	ns
		Orco Modulator Treatment			
		Blank nNMs vs. VUAA0 nNMs	t=0.6123	0.5421	ns
		VUAA0 nNMs vs. VUANT1 nNMs	t=2.372	0.0399	*
		VUAA0 nNMs vs. VUAA4 nNMs	t=3.466	0.0026	**
		Blank NMs vs. VUAA0 NMs	t=0.2879	0.9885	ns
		VUAA0 NMs vs. VUANT1 NMs	t=0.04678	0.9886	ns
		VUAA0 NMs vs. VUAA4 NMs	t=0.1344	0.9886	ns

Volatile Orco Modulator BMOR Bioassay (10-11)	Aggression Index	Kruskal-Wallis Test			
		Kruskal-Wallis Statistic	H=7.373329776	0.0609	ns
Volatile Orco Modulator Mobility Control Bioassay (7-9)	Distance Traveled (cm)	Two-Way Repeated Measures ANOVA (with Geisser-Greenhouse's Epsilon)			
		Interaction	F (6, 56) = 0.7079	0.6445	ns
		Row Factor (Time)	F (1.950, 54.59) = 1.686	0.1954	ns
		Column Factor (Treatment)	F (3, 28) = 0.1265	0.9436	ns
		Subject	F (28, 56) = 14.69	<0.0001	****
		Geisser-Greenhouse	ε=0.9748		
	Time Spent Moving (%)	Two-Way Repeated Measures ANOVA (with Geisser-Greenhouse's Epsilon)			
		Interaction	F (6, 56) = 1.689	0.1406	ns
		Row Factor (Time)	F (1.700, 47.60) = 4.630	0.019	*
		Column Factor (Treatment)	F (3, 28) = 1.058	0.3828	ns
		Subject	F (28, 56) = 9.947	<0.0001	****
		Geisser-Greenhouse	ε=0.8500		
	Rotational Frequency (Count)	Two-Way Repeated Measures ANOVA (with Geisser-Greenhouse's Epsilon)			
		Interaction	F (6, 56) = 1.914	0.0945	ns
		Row Factor (Time)	F (1.877, 52.56) = 2.737	0.0773	ns
		Column Factor (Treatment)	F (3, 28) = 0.5115	0.6776	ns
		Subject	F (28, 56) = 12.89	<0.0001	****
		Geisser-Greenhouse	ε=0.9386		

Appendix D. List of publications

Ferguson ST, Ray A, Zwiebel LJ. "Olfactory Genomics of Eusociality within the Hymenoptera," in G. Blomquist and R. Vogt. (Eds.), Insect Pheromone Biochemistry and Molecular Biology, 2nd Edition, 2020. Amsterdam, Netherlands: Academic Press.

Ferguson ST, Park KY, Ruff A, Bakis I, Zwiebel LJ. "Odor Coding of Nestmate Recognition in the Eusocial Ant Camponotus floridanus," Journal of Experimental Biology, 2020. https://doi.org/10.1242/jeb.215400 (Cover Story)

Ahmed SM, Pitts RJ, **Ferguson ST**, Zwiebel LJ. "Characterization of Chemosensory Responses on the Labellum of the Malaria Vector Mosquito, Anopheles coluzzii," Scientific Reports, vol. 8, no. 5656, 2018. doi: 10.1038/s41598-018-23987-y

Slone JD, Pask GM, **Ferguson ST**, Millar JG, Berger SL, Reinberg D, Liebig J, Ray A, Zwiebel LJ. "Functional Characterization of Odorant Receptors in the Ponerine Ant, Harpegnathos saltator," Proceedings of the National Academy of Sciences, vol. 114, no. 32, pages 8586-8591, 2017. doi: 10.1073/pnas.1704647114