Effects of Odorant-environment Complexity on Behavioral and Neural Plasticity at Different Time Scales

by

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ABSTRACT

The ability to detect and appropriately respond to chemical stimuli is important for many organisms, ranging from bacteria to multicellular animals. Responses to these stimuli can be plastic over multiple time scales. In the short-term, the synaptic strengths of neurons embedded in neural circuits can be modified and result in various forms of learning. In the long-term, the overall developmental trajectory of the olfactory network can be altered and synaptic strengths can be modified on a broad scale as a direct result of long-term (chronic) stimulus experience. Over evolutionary time the olfactory system can impose selection pressures that affect the odorants used in communication networks. On short time scales, I measured the effects of repeated alarm pheromone exposure on the colony-level defense behaviors in a social bee. I found that the responses to the alarm pheromone were plastic. This suggests that there may be mechanisms that affect individual plasticity to pheromones and regulate how these individuals act in groups to coordinate nest defense. On longer time scales, I measured the behavioral and neural affects of bees given a single chronic odor experience versus bees that had a natural, more diverse olfactory experience. The central brains of bees with a deprived odor experience responded more similarly to odorants in imaging studies, and did not develop a fully mature olfactory network. Additionally, these immature networks showed behavioral deficits when recalling odor mixture components. Over evolutionary time, signals need to engage the attention of and be easily recognized by bees. I measured responses of bees to a floral mixture and its constituent monomolecular components. I found that natural floral mixtures engage the orientation of bees' antennae more strongly

than single-component odorants and also provide more consistent central brain responses between stimulations. Together, these studies highlight the importance of olfactory experience on different scales and how the nervous system might impose pressures to select the stimuli used as signals in communication networks.

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INTRODUCTION

Overview

The primary question in my research revolves around the quality of olfactory stimuli (e.g., simple vs complex, pheromone vs general odor cue) and how experiences with these odors shape an animal's behavioral and neural plasticity. This has been broken into three main chapters:

Chapter 2. Does short-term experience affect responses to all odors, including responses to selected signals such as pheromones?

Chapter 3. Does long-term individual experience with odors shape how an olfactory system processes all odors?

Chapter 4. Is the sensory system of pollinators shaped by selection pressures that favor complex odor mixtures over simple, single-component stimuli on flowers?

To address these questions, I studied the responses of social bees to various olfactory stimuli. Below, I summarize current knowledge of the odorant responses of social bees, the olfactory system and its development in bees. I also give an overview of the known mechanisms of experience-driven plasticity in the honey bee antennal lobe.

BACKGROUND

Chemical responses in social insects

Social insects—ants, bees, and other social Hymenoptera—are highly adept at chemical communication and, thus, are ideal for this research (Giurfa, 2012). They can identify a large variety of natural odorants and rapidly coordinate their responses to these odorants in order to appropriately collect food, raise offspring, and defend resources (Billen & Morgan, 1998; Wilson, 1965). Social insects use chemical pheromones to coordinate these behaviors (Billen & Morgan, 1998; Wilson, 1965; Wyatt, 2010). Pheromones innately elicit a stereotyped, state-dependent response, which allows individuals to appropriately behave in a given context (Billen & Morgan, 1998; Blum, 1969; Sasaki, Holldobler, Millar, & Pratt, 2014; Wilson, 1965). Additionally, social insects are capable of responding to many odors, which do not have any innate valence, and can be readily associated with important events in the environment, such as the availability of food or a nest location.

In social insects, pheromones typically elicit modal action patterns, the expression of which is influenced by the social caste and context. These responses are stable within the life of the animal and over evolutionary time (Collins, 1980). Typical social insect pheromones include, but are not limited to, signaling of fertility, alarm, orientation, brood, and caste (Billen & Morgan, 1998; Wilson, 1965). But how social insects learn about alarm pheromones has not been the subject of extensive study. Most studies either focus on the effects of pheromones on learning or how olfactory cues or environmental

conditions affect immediate responses to pheromones (Collins, 1980; Nouvian, Hotier, Claudianos, Giurfa, & Reinhard, 2015; Paxton, Sakamoto, & Rugiga, 1994; Urlacher, Francés, Giurfa, & Devaud, 2010).

On the other side of the continuum, other odors do not elicit these strong modal action patterns (B. H. Smith & Menzel, 1989), but instead can change their valence or associative state rapidly over the life of a single individual. These odors classically include learned resource cues, such as floral odors in bees (Bitterman, Menzel, Fietz, & Schäfer, 1983; Menzel, 1985), but also include predator and other noxious cues (Bray & Nieh, 2014; Carcaud, Roussel, Giurfa, & Sandoz, 2009; Goodale & Nieh, 2012; Lichtenberg, Hrncir, Turatti, & Nieh, 2011; Vergoz, Roussel, Sandoz, & Giurfa, 2007). For an animal to learn about them, these odors must initially elicit 'attention-like' or orienting behaviors. If the odors have inconsistent or less consistent relationships with the important events, the orientation properties of the behavior quickly wane. However, if the odor is associated with something important, such as food, then orienting behaviors increase and are followed by conditioned responses appropriate for the associative connection.

Responses to volatile chemicals in social insects fall along a continuous spectrum from stereotyped modal action patterns, such as responses to pheromones, to orienting behaviors by which animals explore novel odorants. Commonly, studies only use novel odors which initially only elicit orienting behaviors to assess short term behavioral plasticity; odors that elicit modal action patterns are generally only used as a contextual

cue to affect responses to these novel odors (Nouvian et al., 2015; B. H. Smith & Menzel, 1989), with a few exceptions such as Nazonov compounds (Bhagavan & Smith, 1997; Bitterman et al., 1983). However, it is known that responses to volatile chemicals are generally processed through the olfactory system via specific central brain networks—the antennal lobe, lateral protocerebral lobe, and mushroom bodies in invertebrates—and therefore particular olfactory-driven behaviors are subject to the functioning of these brain regions (Erber, Masuhr, & Menzel, 1980; Jakupovic, Kang, & Baum, 2008; Staubli, Schottler, & Nejat-Bina, 1987; Yee & Costanzo, 1995).

In order to explore this idea further, in chapter 2 I use the stingless honey bee, *Tetragonisca angustula*, to measure group level short-term plasticity to their own alarm pheromone. The alarm pheromone is a social signal and therefore it makes the most sense to measure response in a social group context. *T. angustula* is an excellent species to use for such a study, as they have morphologically distinct guards, which perform multiple conspicuous behaviors. I found clear evidence of non-associative plasticity with repeated exposure to their alarm pheromone that returned to a baseline response with increasing time between alarm exposures. This finding supports the idea that pheromones can be thought of as unconditioned stimuli, which fall on the modal action pattern end of the odorant continuum. With repeated exposure unconditioned stimuli responses by animals will generally habituate or sensitize (Mackintosh, 1983), in my case *T. angustula* habituated to the pheromone. I next decided to work with honey bees which have a long

history of odor-dependent effects on behavior in the lab and in the field, as well as a strong body of work exploring the neurobiology of these animals.

Olfactory learning in honey bees

Honey bees, *Apis mellifera*, have long been used in conditioning experiments in research exploring how odors influence foraging and feeding behaviors both in freely flying experiments in the field (Menzel, 1968; Menzel, 1985) and in laboratory experiments using restrained bees (Bitterman et al., 1983; Jernigan, Roubik, Wcislo, & Riveros, 2014). The behaviors of honey bees can be modified through both non-associative and associative experiences (Bitterman et al., 1983; Chandra, Wright, & Smith, 2010; Jernigan et al., 2014). For example, honey bees will increase their responses to odors after sucrose stimulation (Hammer, Braun, & Mauelshagen, 1994) and decrease responses to odors after repeated stimulation without reinforcement (latent inhibition) (Chandra, Hosler, & Smith, 2000; Chandra et al., 2010). In associative assays, odorants (conditioned stimuli, CS) can be paired with unconditioned stimuli (US, appetitive or aversive stimuli) and bees can readily learn, recall, unlearn, and relearn these odorants multiple times throughout their lifetimes (Bitterman et al., 1983; Menzel, 1985; Menzel, 1999; Menzel & Muller, 1996; Rescorla, 1988). This has been demonstrated in association with sucrose in appetitive conditioning experiments (Bitterman et al., 1983; Chandra et al., 2010; B Gerber et al., 1996; Jernigan et al., 2014; Menzel & Erber, 1978; Menzel, Greggers, & Hammer, 1993) or with weak electric shock in aversive conditioning experiments (Cholé, Junca, & Sandoz, 2015; Vergoz et al., 2007). Bees can

also learn operant tasks, in which antennal behaviors will be associated with an outcome (Kisch & Erber, 1999), and bumble bees are even capable of social learning via observation of other bees (Alem et al., 2016).

I was next interested in how long-term experience might affect plasticity and impact the olfactory nervous system and behavior of animals. We know that there is a degree of inter-individual variability in the physiology and protein expression in the olfactory brain centers of bees (Fernandez, Locatelli, Person-Rennell, Deleo, & Smith, 2009; Galizia & Menzel, 2000; Locatelli et al., 2013; I. T. Sinakevitch et al., 2013). In chapter 3, I hypothesized that prior individual experience might drive much of this variability physiologically, morphologically, and behaviorally. Below I will introduce the organization of the olfactory nervous system of honey bees, and discuss what is currently known about the mechanisms that shape the development and plasticity of this brain region.

Organization of the olfactory system in honey bees, *Apis mellifera*

The general path of olfactory information in the honey bee brain is as follows. The olfactory receptor neurons (ORNs) signal the presence of olfactory molecules via electrical information in the form of nerve impulses. These olfactory receptor neurons project their axon terminal outputs to the antennal lobe (AL). The antennal lobe is the primary olfactory processing center in the central brain. Information from the antennal lobe is then transferred via projection neurons (PNs) along several tracts to the lip region

of both calyces of the mushroom bodies, as well as the lateral protocerebral lobe (lateral horn, LH). These regions of the mushroom body are thought to be stimulus coincidence detectors, necessary for associative learning, as well as sensory integration centers necessary for higher-order processing and complex learning tasks observed in honey bees (Brill et al., 2013; J. Carcaud, T. Hill, M. Giurfa, & J.-C. Sandoz, 2012; Galizia & Rössler, 2010; Groh, Lu, Meinertzhagen, & Rössler, 2012; Strube-Bloss, Nawrot, & Menzel, 2011).

Antennae and olfactory receptor neurons

The primary olfactory sensing organs in insects are found on the antennae. The antenna is generally divided into two main regions: the scapus and the flagellum. The flagellum is a segmented region that has multiple types of sensory structures, called sensilla. Sensilla house the dendrites of olfactory receptor neurons and are the site of chemical binding and the beginning of olfactory signaling in the nervous system (Steinbrecht, 1997). There are several types of sensilla, including: Pore plate, trichoid-A, basiconic, coeloconic, ampullaceal, and coelocapitular sensilla (Nishino, Nishikawa, Mizunami, & Yokohari, 2009). Most olfactory sensilla are comprised of pore plate and trichoid-A sensilla (Esslen & Kaissling, 1976; Nishino et al., 2009).

Worker honeybees have approximately 65,000 olfactory receptor neurons, which have their cell bodies housed within the antennae and then project their axons into the antennal lobe via the dorsal and ventral flagellar antennal nerves and four primary tracts (Esslen &

Kaissling, 1976; Galizia & Sachse, 2010; Nishino et al., 2009; Suzuki, 1975). These four tracts are labeled T1 to T4. While T1-T3 more or less receive inputs from both the dorsal and ventral flagellar antennal nerve clusters, the T4 tract contains olfactory receptor neuron axon terminals almost exclusively from the ventral nerve (Nishino et al., 2009).

General antennal lobe organization

Much like the mammalian olfactory bulb, the insect's primary olfactory center is arranged in a glomerular, or a multi-sphere-like, synaptic neuropil (Hildebrand & Shepherd, 1997; I. T. Sinakevitch, Daskalova, & Smith, 2017). The number, size, and arrangement of glomeruli are highly species-specific. However, a few general trends have been observed. Typically, the number of glomeruli strongly correlates with the number of olfactory receptor (OR) genes in insects. Thus, it is believed glomeruli possess a chemotopic organization; in which each glomerulus is only receiving inputs from one type of olfactory receptor neuron (Robertson & Wanner, 2006; I. T. Sinakevitch et al., 2017). In *Drosophila*, 62 olfactory receptor genes have been identified and they possess less than 50 glomeruli. The worker honey bee has approximately 162 olfactory receptor genes and has about 160-170 glomeruli (Fishilevich & Vosshall, 2005; Flanagan & Mercer, 1989a; Galizia, McIlwrath, & Menzel, 1999; Galizia & Sachse, 2010; Robertson & Wanner, 2006). In addition, there is evidence for a somatotopic organization, where by the proximal segments of the antennae project to the outermost cortical region of the glomerulus, and distal segments project to the inner margin of the glomerulus cortical layer (Nishino et al., 2009).

In the honey bee, the antennal lobe is made up of a single layer of glomeruli, which form a spherical structure that has an asynaptic center referred to as the aglomerular neuropil. The glomeruli are very highly organized. The sensory tracts (T1-T4) of the olfactory receptor neurons project their axons to specific glomeruli in the honey bee antennal lobe. The T1 tract innervates approximately 70 glomeruli in the rostro-dorsal region of the antennal lobe; the T2 tract innervates approximately 7 glomeruli along a medially placed band of the antennal lobe; the T3 tract innervates approximately 70 glomeruli along the ventral surface of the antennal lobe; and the T4 tract innervates approximately 7 glomeruli in the caudo-ventral region of the antennal lobe (Arnold, Masson, & Budharugsa, 1985; Flanagan & Mercer, 1989a; Galizia & Sachse, 2010).

Antennal lobe local interneurons

Local neurons (LN) are found to connect glomeruli within the antennal lobe and are distinguished by the uniformity of their connection pattern. The two classes are as follows: homogeneous local neurons (homoLNs) innervate all or most of the glomeruli within the AL, while asymmetric or heterogeneous local neurons (heteroLNs) primarily innervate only a few glomeruli within the antennal lobe (Flanagan & Mercer, 1989b). As would be expected, heterogeneous local neurons are primarily responsive only to the odors that their associated glomeruli respond, while homoLNs show general activity to most odors (Galizia & Kimmerle, 2004; Ng et al., 2002). In honey bees, sodium action potential spiking has been observed in local neurons (Galizia & Kimmerle, 2004; Galizia

& Sachse, 2010). Various local neurons likely act as modulators in the antennal lobe and have been shown to have GABAergic, histaminergic, glutamatergic, and cholinergic staining (Ramaekers, Parmentier, Lasnier, Bockaert, & Grau, 2001; Sachse & Galizia, 2002; Schäfer & Bicker, 1986; Shang, Claridge-Chang, Sjulson, Pypaert, & Miesenböck, 2007). In addition to these neurotransmitters, different types of local neurons have been shown to express or co-express neuropeptides, such as allatotropin, allatostatin, FMRF-amide, RF-amide, and tachykinins (Galizia & Sachse, 2010; Homberg & Müller, 1999; Iwano & Kanzaki, 2005; Nässel, 1993; Schachtner, Trosowski, D'Hanis, Stubner, & Homberg, 2004).

Antennal lobe projection neuron tracts

Projection neurons receive inputs from olfactory receptor neurons, local neurons, and other projection neurons, and project their axons from the antennal lobe to higher-order brain centers such as the mushroom bodies (MBs) and protocerebrum (lateral horn) of the insect brain (Brill et al., 2013; J. Carcaud, T. Hill, M. Giurfa, & J. C. Sandoz, 2012; Galizia & Rössler, 2010). There are two types of projection neurons: uniglomerular projection neurons (uPNs) and multiglomerular projection neurons (mPNs). As their names suggest, uniglomerular projection neurons primarily innervate a single glomerulus in the antennal lobe and project to the mushroom bodies as well as the lateral horn, primarily via the median-antennoprotocerebral tract (m-APT) or the lateral-antennoprotocerebral tract (l-APT) (Abel, Rybak, & Menzel, 2001; Kirschner et al., 2006). On the other hand, multiglomerular projection neurons innervate most, if not all,

of the glomeruli of the antennal lobe. Additionally, multiglomerular projection neurons primarily project to the ring neuropil around the vertical lobe and lateral protocerebral lobe (LPL), but not to the mushroom bodies, via the several types of mediolateral-antennoprotocerebral tract (ml-APT) (Abel et al., 2001).

The lateral and medial-antennoprotocerebral tracts form a "dual" or "parallel" olfactory pathway, innervating the same regions but running in opposite directions (Brill et al., 2013; Galizia & Rössler, 2010; Kirschner et al., 2006). The lateral-antennoprotocerebral tract is innervated primarily by the T1 antennal nerve tract glomeruli and projects laterally in the brain in a stereotypical pattern to the lateral protocerebral lobe and lateral horn and then to the lip region of each of the mushroom body calyces (Abel et al., 2001; Galizia & Sachse, 2010; Kirschner et al., 2006). The medial-antennoprotocerebral tract is innervated primarily by the T3 antennal nerve tract glomeruli and projects medially, first to each of the lip regions of the mushroom body calyces, then to the lateral protocerebral lobe and the lateral horn (Abel et al., 2001; Galizia & Sachse, 2010; Kirschner et al., 2006). The lip region of the mushroom body calyces receives segregated inputs from the lateral- and medial-antennoprotocerebral tracts. The information carried by each tract is suggested to be separately maintained in the mushroom bodies (Abel et al., 2001; Kirschner et al., 2006). It is currently unknown if each of these two tracts carries different information.

There are generally 3 described types of the mediolateral-antennoprotocerebral tracts (ml-APTs). Two of these (ml-APT2 and ml-APT3) project to the lateral protocerebral lobe and vertical lobe of the mushroom bodies, while the third (ml-APT1) projects its axons primarily to the lateral horn (Abel et al., 2001; Kirschner et al., 2006). The mediolateral-antennoprotocerebral tract neurons have been shown to be GABAergic, at least around the vertical lobe (Bicker, Kreissl, & Hofbauer, 1993; Kirschner et al., 2006). The exact function of these tracts is still an ongoing area of research.

Development of the antennal lobe

The adult antennal lobe begins formation during prepupal stages and is highly stereotyped between individuals (Flanagan & Mercer, 1989a; Cosmas Giovanni Galizia et al., 1999). The formation of glomeruli within the antennal lobe requires input from olfactory receptor neurons during late larval and prepupal stages (Hähnlein & Bicker, 1997; Hildebrand, Hall, & Osmond, 1979; Oland, Orr, & Tolbert, 1990). If you remove the antenna during pupal development, the antennal lobe develops with reduced volume and an aglomerular structure (Gascuel, Masson, & Beadle, 1991; Hildebrand et al., 1979; Oland & Tolbert, 1987). Olfactory receptor neuron axons project to the antennal lobe and an asynaptic preglomerulus (protoglomerulus in *Manduca*) forms, surrounded by a sheath of glial cells (Gascuel et al., 1991; Oland et al., 1990; Oland & Tolbert, 1987, 1989). This preglomerulus defines the area in which the glomerular synaptic neuropil will develop. Projection neurons will next arborize within these preglomeruli, prior to mature synaptic formation (Hähnlein & Bicker, 1997; Oland et al., 1990; Schröter & Malun, 2000). The

adult synaptic glomeruli can later be visualized at the last stage of development when mature synapses form between olfactory receptor neurons, projection neurons, and local neurons (Hähnlein & Bicker, 1997; Oland et al., 1990; Oland & Tolbert, 1989; Schröter & Malun, 2000).

Higher-order olfactory brain centers

The mushroom bodies are the secondary sensory integration centers of the protocerebrum of the insect brain and have been shown to be involved with complex learning tasks and recall in honey bees (Strube-Bloss et al., 2011; Szyszka, Galkin, & Menzel, 2008). The mushroom bodies are sensory integration centers, receiving both olfactory and visual input in most insects (Farris, 2005; Strausfeld, Hansen, Li, Gomez, & Ito, 1998). The calyces of the mushroom bodies of Hymenoptera are divided into the lip, collar, and the basal ring regions, which also correspond to layers in the vertical lobe. As discussed above, the lip region primarily receives input from the olfactory centers. The collar region lies between the lip and the basal ring, and receives primarily visual inputs (Galizia & Sachse, 2010). The basal ring region receives both olfactory and visual inputs (Galizia & Sachse, 2010).

The protocerebrum is comprised of the lateral protocerebral lobe, the lateral horn, and the vertical lobes of the mushroom bodies. All of these regions and the mushroom bodies receive input from the antennal lobe via the antennoprotocerebral tracts as discussed above. However, only the mushroom bodies and the lateral horn have been successfully

segregated by input (Ehmer & Gronenberg, 2002; Galizia & Sachse, 2010; Knaden, Strutz, Ahsan, Sachse, & Hansson, 2012; Ruta et al., 2010; Rybak & Menzel, 1993; Strutz et al., 2014). The mushroom body lip region has defined input regions from both the lateral and medial-antennoprotocerebral tracts, most of which appear to be cholinergic (Abel et al., 2001; Hammer & Menzel, 1995). The lateral horn also has segregated compartments; Kirshner et al. (2006) suggest there are at least four compartments. However, they could only detect that compartment #1 receives only medial-antennoprotocerebral tract input and that compartment #3 does not receive medial-antennoprotocerebral tract input (Kirschner et al., 2006).

Mechanisms of odor-driven plasticity in the honey bee antennal lobe

The antennal lobe is an important locus of associative and non-associative plasticity in addition to being a hub for odor encoding (Fernandez et al., 2009; Locatelli, Fernandez, & Smith, 2016). The antennal lobe therefore participates in not just odor discrimination, but also in association and recall (Farooqui, Robinson, Vaessin, & Smith, 2003; Sachse & Galizia, 2002). The odor-driven antennal lobe activity shows characteristic changes in the glomerular responses to an odor mixture, which shifts its odor response patterns and makes these patterns more discriminable from other mixtures that have not been associated with appetitive rewards (Fernandez et al., 2009). Likewise, there are characteristic changes in the antennal lobe that shift mixture responses to be more different from unreinforced odor patterns in mixtures (Locatelli et al., 2013). It is likely that this plasticity is derived from modifications in the network via inhibitory—

GABAeric—local neurons, which can modify the activity outputs of both other GABAergic local neurons and projection neurons to higher-order brain regions (Locatelli et al., 2013; Rein, Mustard, Strauch, Smith, & Galizia, 2013).

GABAergic local neurons interconnect glomeruli in the antennal lobe, mediating lateral and global inhibition (Barbara, Zube, Rybak, Gauthier, & Grünewald, 2005; Sachse & Galizia, 2002). Local neurons project to multiple glomeruli within the antennal lobe, modulating the activity of neighboring glomeruli (Rein et al., 2013; Sachse & Galizia, 2002). Another important signaling molecule in the antennal lobe, octopamine, has been found to signal the presence of reward (e.g. sucrose) in the antennal lobe during appetitive learning (Hammer & Menzel, 1998). Octopamine is released from the ventral-unpaired median neurons and other less-studied memory centers of the honey bee brain during olfactory learning (Hammer, 1993; Hammer & Menzel, 1998; Kreissl, Eichmüller, Bicker, Rapus, & Eckert, 1994).

Direct pairing of odor with octopamine microinjection into the calyces of the mushroom bodies or the antennal lobe is sufficient to induce appetitive conditioning to the odor without the use of an appetitive reward (Hammer & Menzel, 1998). Furthermore, the disruption of an octopamine receptor—AmOA1—in the antennal lobe can disrupt olfactory learning and recall (Farooqui et al., 2003; Farooqui, Vaessin, & Smith, 2004). AmOA1 is also primarily expressed in the GABAergic local neurons of the antennal lobe and has been shown to modulate Ca^{2+} in the cell (Grohmann et al., 2003; I. Sinakevitch,

Mustard, & Smith, 2011; I. T. Sinakevitch et al., 2013). Octopamine seems to make odors associated with rewards more discriminable via this change in the odor's representation in the antennal lobe (Chen et al., 2015; Fernandez et al., 2009; Locatelli et al., 2013; Rein et al., 2013). Together these findings provide evidence that AmOA1 may act as a key modulator driving the plasticity of the olfactory network of the honeybee.

Lastly, foraging experience has been associated with volumetric changes in glomeruli within the antennal lobe (Brown, Napper, & Mercer, 2004; Brown, Napper, Thompson, & Mercer, 2002) and volumetric changes in the mushroom bodies (Fahrbach, Farris, Sullivan, & Robinson, 2003; Fahrbach, Giray, & Robinson, 1995; Fahrbach, Moore, Capaldi, Farris, & Robinson, 1998; Farris, Robinson, & Fahrbach, 2001), however, the exact neuronal subpopulations involved are not yet known. Broad experience-dependent modulation may also act through novel synapse formation and some of the mechanisms described above, however these ideas have yet to be supported experimentally.

It is clear that experience, either at longer developmental time-scales or at shorter time scales, shapes the brain through some of the processes discussed above. As a first step to understand this plasticity, in chapter 3, I discuss the impacts of long-term olfactory experience in a cohort of bees that had access to only a single foraging odor on inter-individual plasticity. I found that reduced experience reduced the inter-individual variability in how the antennal lobe responds to odors, delayed the developmental trajectory of the antennal lobe, and caused deficits in odor mixture processing.

Floral odorant diversity in the environment

This last finding led me to question what is unique about odor mixtures? Complex mixtures both take more experience on the part of the pollinator to process and more energy to produce on the part of the flower, so why are they so common in floral signals? The processing of odor mixtures is crucial for a pollinator's success. The nervous system of a generalist pollinator needs to be able to quickly learn and recall floral mixture bouquets. This allows them to maintain flower constancy, foraging at the same floral species over time. Resources allow pollinators to grow and provide for offspring, and, in the case of honey bees who store resources, it enables them to survive the flowerless winter. Additionally, the most successful honey bee colonies must forage at the most profitable floral resources at any given time. As a result, honey bees show strong learning capabilities, which allow them to both maintain this flower constancy as well as learn to switch floral species as a more profitable source becomes available (B Gerber et al., 1996; Christoph Grüter, Moore, Firmin, Helanterä, & Ratnieks, 2011; Heinrich, Mudge, & Deringis, 1977; Menzel, 1985).

Typically, floral odorants that signal a rewarding flower to the bees contain multiple chemical components, which can vary in both intensity and ratios between inflorescences within a single species (Knauer & Schiestl, 2015; Wright & Schiestl, 2009). In order to learn from these signals, honey bees must be initially attracted to these floral odors, then recognize the floral mixture bouquet from the same species with a known variability, and also recall the floral odorant mixture in order to maintain flower constancy.

Despite the fact that there are many chemicals produced by any given rewarding florescence, when bumble bees are allowed to forage on a the common canola oil flower, *Brassica rapa*, they develop a preference in particular for one component—phenyl acetaldehyde (Wright & Schiestl, 2009). If this is the case, why are floral odorant stimuli so complex, and might the behavior and olfactory nervous system of bees and other pollinators act as a selection pressure to maintain this odor complexity?

In chapter 4, I explore why floral odors are generally comprised of many chemical components from the perspective of the honey bee sensory nervous system and antennal behavior. I measure the antennal behaviors of honey bee foragers in response to the floral mixture bouquet of *B. rapa* and its constituent components. I also measure the antennal lobe output responses to these odors to see if there are any sensory or behavioral preferences for floral mixtures that might explain why natural floral odors are generally so complex. I found that there is a strong trend that the mixtures are both more behaviorally stimulating to honey bees and more consistently signaled in the antennal lobe. Both of these effects could provide strong selection pressures on floral species to produce mixtures rather than single components, even if some bees may only be responding to one chemical component when associating the floral bouquet with a sucrose reward.

SIGNIFICANCE

Experience with odors shapes both behavior and the neurobiological networks that process odors on multiple time scales. In this dissertation work, I provide additional support for this statement in social bees at short time scales. Chapter 2 shows that responses to novel odors and pheromones can be modified by individual experience. Chapter 3 provides a new foundation for understanding how post-eclosion olfactory experience shapes the brain over long time scales. A diversity of olfactory experience as an adult is necessary for full antennal lobe maturation and normal olfactory behavior in honey bees. Chapter 4 shows that multi-component, complex-odor mixtures provide higher levels of behavioral stimulation and more consistent intra-glomerular responses between stimulations. This result suggests that bees actively seek out complex odors, which will, in turn, be easier to recall by the nervous system and perhaps also provide them with a larger diversity of odor experience and facilitate the maintenance of a mature antennal lobe network. Together this work improves understanding of odor-driven plasticity in bees at both short and long-term time scales. It also provides evidence that the olfactory nervous systems of pollinators currently provides a strong selection pressure on flowering plants to produce multi-component mixtures associated with their inflorescences.

CHAPTER 2

COLONY-LEVEL NON-ASSOCIATIVE PLASTICITY OF ALARM RESPONSES IN A STINGLESS HONEY BEE

Abstract

In ants, bees, and other social Hymenoptera alarm pheromones are widely employed to coordinate colony nest defense. In that context, alarm pheromones elicit innate species-specific defensive behaviors. Therefore, in terms of classical conditioning, an alarm pheromone could act as an unconditioned stimulus (US). Here we test this hypothesis by establishing whether repeated exposure to alarm pheromone in different testing contexts modifies the alarm response. We evaluate colony level alarm responses in the stingless bee, *Tetragonisca angustula*, which has a morphologically distinct guard caste. First, we describe the overall topology of defense behaviors in the presence of an alarm pheromone. Second, we show that repeated, regular exposure to synthetic alarm pheromone reduces different components of the alarm response, and memory of that exposure decays over time. This observed decrease followed by recovery occurs over different time frames and is consistent with behavioral habituation. We further tested whether the alarm pheromone can act as a US to classically condition guards to modify their defense behaviors in the presence of a novel (conditioned) stimulus (CS). We found no consistent changes in the response to the CS. Our study demonstrates the possibility that colony-level alarm responses can be adaptively modified by experience in response to changing environmental threats. Further studies are now needed to reveal the extent of these habituation-like responses in regard to other pheromones, the potential mechanisms

that underlie this phenomenon, and the range of adaptive contexts in which they function at the colony level.

INTRODUCTION

Highly social insects, such as honeybees (Apini*)* and stingless honey bees (Meliponini), build large colonies that provide for brood rearing and storage of resources. Colonies collect resources scattered over the environment and concentrate them in one place for rearing brood and survival though changes in seasons (Hansell, 1993; McGlynn, 2012; Wilson, 1971). Concentrated stores of brood and resources are valuable sources of carbohydrate and protein, which many animals, including other social insects, attempt to exploit. Therefore, a social central-place lifestyle necessitates development of a rapid and coordinated colony defense. Social insects have evolved some of the most sophisticated mechanisms known for communication and coordination of defensive behavior.

In ants, bees, and other social Hymenoptera, alarm pheromones (AP) are widely employed to coordinate nest defense against both conspecifics and heterospecifics (Blum, 1969). Alarm pheromones are a common class of pheromones, which elicit defensive and escape behavior in social insects (Blum, 1969; Ulrich Maschwitz, 1964). The nature of the response in any individual depends upon its caste and the context in which the alarm pheromone is presented (UW Maschwitz, 1967; Sasaki et al., 2014; Shorey, 1973). At or near the colony, alarm pheromones elicit defensive behaviors, especially among specialized guard castes (Blum, 1969; UW Maschwitz, 1967; Sasaki et al., 2014; Wilson,

1965). Classically, honeybees sacrifice themselves in colony defense against mammals through stinging and subsequently eviscerating themselves to defend the colony (Hermann, 1971). A similar sacrificial behavior has been described in several species of stingless honey bees, in which individuals perform a "death-grip" on an object that poses a threat, and those individuals typically stop moving shortly after performing this behavior (**Figure 2.S1**) (personal observation; (Shackleton et al., 2015). In addition to extreme but relatively rare forms of defense, there are more common defensive behaviors that can be more consistently observed across a range of the stingless honey bees. Those include biting, presenting visible guard forces at the nest entrance, application of resins, caustic chemicals and/or toxins to invaders, and reducing the nest entrance size (Butler & Free, 1951; Hammel et al., 2016; Roubik, 1992, 2006; Sakagami, Roubik, & Zucchi, 1993).

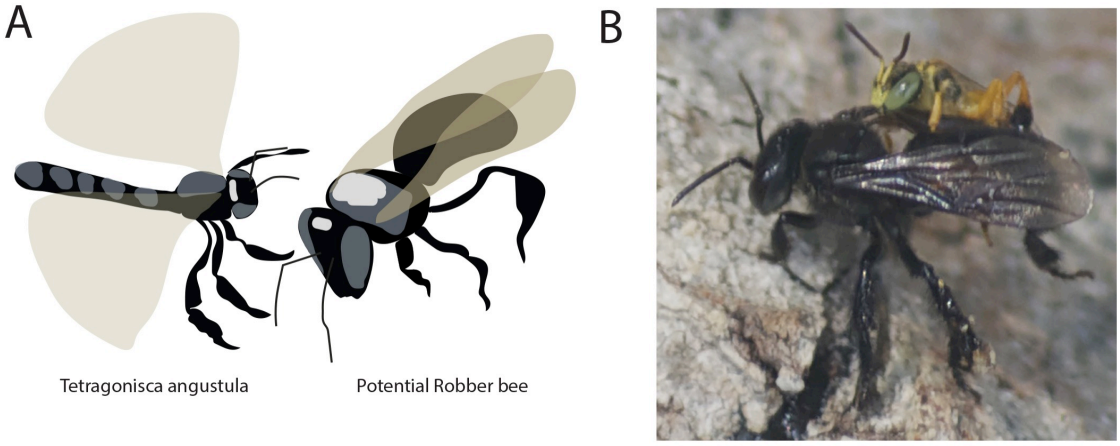


Figure 2.S1. **A**. Diagram of a lunging attack by a *Tetragonisca angustula (*left) guard onto a potential robber bee (right) presented to the hovering guard. **B**. A photograph of *T. angustula* (upper-right), which performed a 'death grip' on the wing of a stingless bee

raider (*left*, *Trigona sp.*). The potential raider was observed near the *T. angustula* nest and was likely scouting to recruit a raid on the *T. angustula* nest.

When social bees are presented with their species-specific alarm pheromone, they consistently and readily perform defensive behaviors. However, the response incurs a cost via energy expenditure, disruption of foraging activity and potential death it causes to conspecifics. Therefore, it could hypothetically be the case that a truly adaptive response would require behavioral adaptation to match the level of threat. For example, defensive behavior might be reduced in response to repeated low-level threats, such as disturbance via a single non-nestmate scout bee. Alternatively, defensive behavior might be augmented in response to repeated, high-level threats such as a multi-individual raid or attempted raid on the nest in which there is a threat of significant loss of colony resources or workforce. Furthermore, it might be beneficial to learn the pairing of alarm pheromone with the specific odors of threatening species, such as kairomones, so that the response could be adjusted more reliably in future encounters. Thus the alarm pheromone defense system in social insects might be easily adapted to different situations using non-associative and associative conditioning.

In terms of classical conditioning, a pheromone is an Unconditioned Stimulus (US) (Martínez-Ricós, Agustín-Pavón, Lanuza, & Martínez-García, 2006; Yunker, Wein, & Wisenden, 1999). In a motivated animal, a US elicits an innate response that is performed in a consistent and stereotypic manner (Mackintosh, 1983; Rescorla, 1988). Typically,

the response to the US can be modified by non-associative mechanisms. For example, repeated exposure to a US reduces the response through habituation (Rescorla, 1988; Thompson & Spencer, 1966). A US can also produce sensitization to other stimuli. Furthermore, a US can be associated with another stimulus – a Conditioned Stimulus (CS) – and modify the response to the CS in a way that is consistent with the pairing (Mackintosh, 1983).

Here we test hypotheses about colony-level experience-based modification of the alarm pheromone response in the stingless honey bee, *Tetragonisca angustula*, which is common in South and Central America. The nests of *T. angustula* have a single tube-like defense structure with a single nest entrance/exit, patrolled by morphologically distinct guards (Christoph Grüter, Menezes, Imperatriz-Fonseca, & Ratnieks, 2012; Christoph Grüter, Segers, Menezes, et al., 2017; Christoph Grüter, Segers, Santos, et al., 2017; Hammel et al., 2016). These guards perform two types of guarding behaviors, standing and hovering. Standing guards remain on and near the nest entrance tube, and are thought to defend the entrance against conspecific raiders (Kärcher & Ratnieks, 2009; van Zweden, Grüter, Jones, & Ratnieks, 2011). Another group of guards constantly hovers in place near the nest entrance, defending primarily against airborne heterospecific raiders (Bowden, Garry, & Breed, 1994; C Grüter, Kärcher, & Ratnieks, 2011; Sakagami et al., 1993; van Zweden et al., 2011; D Wittmann, 1985; Dieter Wittmann, Radtke, Zeil, Lübke, & Francke, 1990). Using those two populations of guard bees and the forager population, we specifically test the hypothesis that a pheromone can be treated as and

unconditioned stimulus. We would predict that repeated exposure can modify the colony-level response to the alarm pheromone, and that memory of the exposures decays over time. Furthermore, we test whether associations of the *T. angustula* alarm pheromone with the kairomones of other stingless honey bees, which typically attack and raid *T. angustula* colonies, can modify the colony-level responses exhibited by *T. angustula* colonies to kairomones. Such a result would be consistent with associative conditioning and possibly be a first mechanistic step to establishing consistent neighbor relationships in dense social insect environments.

METHODS

General experimental procedure

Tetragonisca angustula colonies were observed using a dual camera setup in Gamboa, Colón, Republic of Panama from April to August of 2013. The two cameras were set up with one as a zoom on the *T. angustula* nest entrance and the second as a wide view to capture a 1m radius around the nest entrance. All olfactory stimuli were presented near the nest entrance on a small circular disk of filter paper with a 7 mm diameter pinned to a black sponge (B. Smith & Roubik, 1983). The sponge was used as a visual target, the filter paper and chemicals were not in direct contact with the sponges. Black color has previously been shown to facilitate high levels of defensive response in *T. angustula* (Bowden et al., 1994). Most interactions were with the filter paper, which was newly prepared for each presentation. As an additional control, after each use sponges were hand washed 4-5 times using an odorless laundry detergent. They were then rinsed with tap water for several hours (20-40 rinses). We performed a Pearson correlation analysis

of the behavioral responses over time to test if this washing procedure was sufficient, or the response strength was increasing or decreasing over the time course of experiments using the same sponges and found no effect over time on response rates to the reuse of sponge controls.

We had access to a total of 16 *T. angustula* colonies over the course of all experiments; 6 colonies kept in man-made hive boxes, and 10 naturally nesting colonies of similar size and were accessible to our setup. Some of the colonies were only used in one experiment, however some were used in multiple experiments. In the latter case, we waited over 30 days between experiments on any colony. According to Grosso and Bego (2002), the adult life span of worker *T. angustula* is near 21 days, making it unlikely that any individuals in a colony experienced more than one experimental treatment(FERREIRA GROSSO & Bego, 2002). Thus all bees were likely naïve to stimuli upon first exposure. We performed non-destructive tests and therefore do not know the exact size of each colony. However all colonies compared in our analyses were well established and maintained a constant worker output and very similar numbers in both hovering and standing guards at the nest entrance when undisturbed. All of these tests are transient behavioral responses generally lasting less than 15 minutes. During raid events colonies will remain in a highly defensive state for multiple days (personal observation). Thus we are only testing changes in sensitivity to these stimuli rather than a more taxing whole colony mobilization.

All colonies were observed for 2-3 days prior to any experiment to establish baseline activity. An experiment began by observing activity for 2-3 mins just prior to each manipulation. Next, a filter paper control disk with only mineral oil was presented on the black sponge for 3 mins prior to all chemical presentation. Treatment stimuli (natural or synthetic alarm pheromone) were then presented on the black sponge and activity was observed for 10 mins. After the treatment stimulation, a 3-min observation without any chemical stimulus was video-recorded, starting approximately 1-2 mins after chemical stimulus observation.

Natural alarm pheromone stimulus preparation and presentation

Six colonies were observed for responses to natural alarm pheromone between May and June 2013 in Gamboa, Panama. Observation sequence as described above. The natural alarm pheromone was presented using a non-nestmate guard bee. The bee was captured in a vial and then its head was crushed onto a filter paper disk, using forceps to release the alarm pheromone from the mandibular glands (B. Smith & Roubik, 1983).

Stimulation with synthetic chemical alarm pheromone

We shifted to a synthetic alarm pheromone to ensure observed results were specifically a response to alarm pheromone stimulation rather than to other possible compounds released from crushed heads. The synthetic alarm pheromone mixture was diluted to 1% chemical by volume in mineral oil. The synthetic alarm pheromone was composed of: citral (Sigma Aldrich, B1334), 6-methyl-5-hepten-2-one (Sigma Aldrich, M48805), and

benzaldehyde (Sigma Aldrich B1334). Benzaldehyde has been identified in chemical analyses and behavioral studies as part of the alarm pheromone of *T. angustula* (see Wittmann et al. 1990 for discussion of *T. angustula* alarm profile). The former two components are also known components of the mandibular gland secretion of the robber stingless bee *Lestrimelitta limao,* and therefore could act as kairomones, which also behaviorally elicit defensive behaviors in *T. angustula* (Francke et al., 2000; Dieter Wittmann et al., 1990). All chemicals were stored in a refrigerator at approximately 4°C when not in use, and fresh chemical solutions were prepared from a stock solution each week.

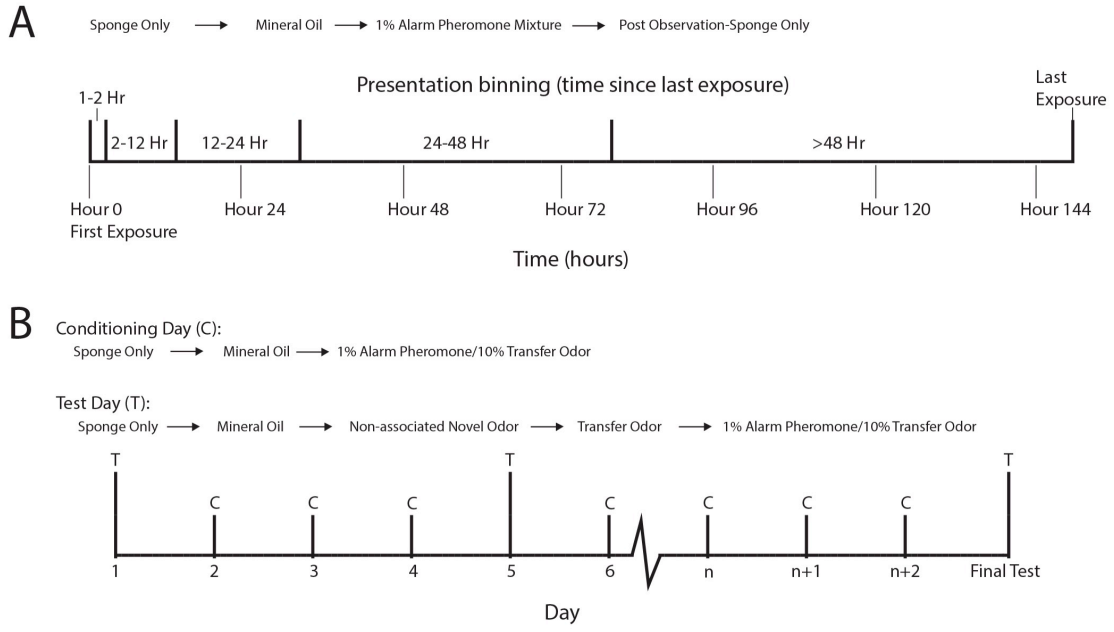


Figure 2.1. A. Time line for stimulus presentation during stimulation with synthetic alarm pheromone. The above vertical lines along the time line represent times at which a colony was presented with alarm stimuli. During each alarm stimulation (vertical line), we presented and collected data in the following sequence: first with visual target-sponge

only, second mineral oil on visual target, third 1% synthetic alarm pheromone mixture (AP), and last a post observation of the visual target alone. The bottom vertical lines represent continuous time in hours of the experiment. **B.** Time line for classical conditioning to a novel odor alarm association experiment. The vertical lines along the time line represent stimulus presentation. There were two types of stimulus presentation, conditioning days (C) and test days (T). On conditioning days colonies were presented with stimuli in the following order: sponge visual target only, then mineral oil on visual target, and last the 1% synthetic alarm pheromone (AP) and 10% transfer odor in mineral oil on two separate filter paper pieces. On test days, stimuli were presented separately and then paired to determine if there is altered behavior to novel transfer stimuli alone. We presented stimuli in the following order on t days: sponge visual target only, mineral oil on visual target, non-AP-associated novel odor on visual target, AP-associated novel odor on visual target, and finally the paired AP and AP-associated novel odor together on visual target.

Nine previously un-manipulated, completely naïve colonies were exposed to alarm pheromone 6-7 times over the course of 7 days (**Figure 2.1A**) in June and July 2013 in Gamboa, Republic of Panama. Colonies were observed for two days prior to the start of experiments to get a baseline of colony behavior before the onset of experiments. Then the colonies were tested using the general procedure described above. Colonies were only tested when there was no rain and little wind, because bees generally reduced their activity during adverse weather. Following the pre-experimental observation and mineral

oil presentation steps, colonies were exposed to a 1% synthetic chemical alarm pheromone mixture (1% Citral, 1% 6-methy-5-hepten-2-one, and 1% benzaldehyde, v/v) in mineral oil and videotaped for 10 mins. On the first day of experimentation, five colonies were exposed to testing for the first time in the morning and four colonies were exposed to testing for the first time in the early afternoon. On the first day of alarm pheromone stimulation colonies were tested 3 times. Two sets of colonies were established. The testing times for the second set was shifted by approximately 2-3 hours later than the first as a control for circadian changes in defensive behavior. Both sets of colonies were then exposed for the second time approximately 1 hr after the first exposure and then the third time approximately 2 hrs later in the mid- or late-afternoon. The colonies were tested again over subsequent days with increasing time intervals between stimulations approximately 14, 38, 86 and 158 hrs later. In sum, these intervals correspond to approximately 1 hr, 2 hrs, 12 hrs, 24 hrs, 48, and >48 hrs since previous exposure (see **Figure 2.1A** for diagram of experimental timeline). Video observations were collected on 56 different occasions, 32 of which were associated with chemical alarm pheromone exposure and 24 of which were prior to experimental onset. Thus we collected a total of 336-560 min (not all pre exposure videos were fully quantified as we collected data for 3 days before and after all treatments) of observational video without chemical alarm pheromone and approximately 310 min of experimental video with chemical alarm pheromone.

Classical conditioning to a novel odor association with alarm pheromone

Five colonies were tested using the colony-level responses to the association of a novel odor with synthetic alarm pheromone (**Figure 2.1B**) between July and August 2013 in Gamboa. Colonies were presented with alarm pheromone and odor pairings once every 24 hrs to avoid sensory adaptation or habituation. We used two novel compounds: 3-heptanol (Sigma Aldrich, W354708) and N-octane (Sigma Aldrich, 412236). Novel odor stimuli were diluted to 10% by volume in mineral oil. Three of the colonies experienced 3-heptanol paired with alarm pheromone and the other two experienced N-octane paired with alarm pheromone. For each type of colony, the remaining odor served as an unpaired novel odor comparison (see below).

We defined two different types of experimental days: conditioning days (C) and Test days (T) (**Figure 2.1B**). During conditioning days, colonies only had presentations of mineral oil alone (3 min) followed by the pairing of alarm pheromone and the paired odor (either 3-heptanol or n-octane; Fig 2.1B). Test days occurred on the 1st day, the 5th day, and every subsequent 5th day afterwards throughout the experiment, while conditioning days occurred during the 4 days in between tests. On test days colonies were sequentially presented with the following stimulus sequence and timing (**Figure 2.1B**): mineral oil (3 min); the *unpaired* odor alone (10 min); the alarm pheromone-*paired* odor alone (10 min); and then the latter odor and alarm pheromone presented together (10 min). There was an interval of 3 mins between each stimulus presentation. Test days were used to compare responses to the *paired odor* and the *unpaired odor* starting before conditioning

and then periodically after conditioning days. All colonies were presented with both odors as a control against changes in behavior due to repetition and not to the pairing procedure. The odors were presented in a non-random order, as we were actively conditioning the colonies on all days and sought to avoid inhibitory effects due to the novel odor not reliably predicting alarm pheromone once every 5 days. Responses on test days were videotaped for offline analysis of behavioral changes to odors. Each colony was measured over a period of 20 days, for a total of 5 test days.

Video Analyses

Video data were collected blind to experiment and experiment start date. However, observers were aware of stimulus presented in each video so that the odor identity could be audibly recorded in the video. We defined several parameters related to colony behavior, which we could quantify in videotape playbacks: rate of bees moving into and out of the entrance; number of landing attacks on the sponge visual target; and a general colony activity measure (see **Table 2.1** for full descriptions). We used these parameters to quantify colony-level behaviors. Videos were analyzed using hand counts with an observer over 3-min windows. All videos allowed for identification and counts of foragers exiting and entering the nest as well as the number of bees that attacked the stimulus (**Table 2.1**). Numbers of both standing and hovering guards were visually counted at the time of recording, and these numbers were later re-confirmed using wide-view video recordings. All videos were analyzed twice by at least two different individuals to confirm counts were accurate and consistent.

Table 2.1. The list of behavioral terms used and description of behaviors/measurements those terms refer to.

Behavioral Term	Description of Behavior
Attacks	Short range aggressive pursuit behaviors directed toward either sponge or olfactory stimuli. The bees fly in a pattern with head oriented toward the stimulus then a bee raises the legs toward stimulus and quickly fly towards and grip stimulus. This behavior is often, but not always, followed by the bee biting the stimulus with its mandibles. This is a quantification of these observed attacks or aggressive pursuit and grip behaviors.
Number Attacking	This is an estimate of the number of bees that are performing the attacks. This measure was done to ensure that behaviors were truly colony level behaviors and not due to one or a few very stimulated individuals.
Rate in	The rate of bees entering the nest via the nest tube.
Rate out	The rate of bees exiting the nest via the nest tube.
Net Influx rate	The difference between the rate of bees entering the nest and the rate of bees exiting the nest. Thus a positive number indicates more bees are entering than exiting, and a negative number indicates more bees are exiting than entering.
Activity	A measure calculated using the SwarmVision software (Birgiolas et al. 2016) to quantify pixel changes over a one min window within a video. In all cases we used a relative change between paired observations by subtracting the pixel changes during mineral oil stimulation from the pixel changes during synthetic alarm pheromone stimulation. We did this to ensure we account for variation in hive size and overall activity between nests.

We assessed overall colony activity using SwarmSight software (Birgiolas, Jernigan, Smith, & Crook, 2017); Table 1). SwarmSight analyzes pixel changes between frames over a set time. We used SwarmSight to simultaneously quantify pixel changes during the mineral oil presentation and the alarm stimulus presentation. These bouts were filmed within 10 mins of each other from the same angle and distance, and we only collected these data when wind and other factors did not affect observed movement (see Birgiolas et al. 2017 for further information). Thus we could use the mineral oil as a baseline control to quantify potential activity changes in the responses to alarm pheromone treatments. We began analysis 30 seconds after stimuli were presented and quantified

pixel changes for 1 min. Hereafter, all mention of activity refers to the above method of pixel change quantification and analysis.

Statistical Analyses

All statistical analyses and plots were made with the R statistics package (R Core Team, 2016). We calculated the colony rates for all forager movements, counts for guard behaviors, and overall colony activity, by subtracting values measured during mineral oil presentation from those measured during alarm stimulation (**Table 2.1**). These behavior changes were then compared over time using repeated measures ANOVA, while significant stimuli/observation differences were determined using the Tukey HSD test. All ANOVA tests used colony as a factor, to ensure we accounted for any colony level differences. Time binning for repetitive exposure experiments was determined by the time since previous alarm simulation. This was done by calculating the time since previous alarm stimulation and adding a binning factor in R using the following categories: first alarm pheromone exposure, less than 2 hrs, 2-12 hrs, 12-24 hrs, 24-48 hrs, and greater than 48 hrs since previous alarm pheromone exposure (**Figure 2.1A**). The large windows were due to differences in the onset of first exposure (as stated above some colonies began stimulation in the morning and others began in the afternoon to control for any circadian effects). In order to test if circadian patterns may have an impact on our data, we ran a separate set of ANOVA tests in which we only considered time of day, either morning or afternoon, and colony as factors. We considered a measurement during the morning if observations were made before 12:00 PM, and all observations

made after 12:00 PM were considered as afternoon. Additionally, we also performed a GLM analysis and model fitting tests of the same data, which can be found in the supplemental section. These analyses showed the same trends as the presented ANOVA tests (**Figure 2.S5**).

RESULTS

Topology of the defensive response to alarm pheromone

Tetragonisca angustula colonies differed in forager activity and number of guards. However, the visual target used for the assays did not affect any behavioral measures. There were no differences in forager movement rates caused by the presence of the target, as measured prior to and during presentations (mean ± SE for: *rate in* target 16.974 ± 2.848, no target 17.595 ± 2.265, ANOVA, F-value=0.029, df=1, p=0.866; *rate out* target 15.128 ± 2.987, no target 12.366 ± 2.062, ANOVA, F-value=0.579, df=1, p=0.454; *net influx* target 1.846 ± 1.478, no target 5.228 ± 1.819, ANOVA, F-value=2.081, df=1, p=0.162; summed over all experiments). Furthermore, we never observed attacks (**Table 2.1**; **Figure 2.S1A**) on the target in the absence of synthetic or natural alarm pheromone (AP).

Forager Movement: There was no effect of stimulus on the rate of bees entering the nest across treatments (ANOVA, F-value=0.815, df=5, p=0.5403, **Figure 2.2A**). However, there was an effect on the rate of bees exiting the nest (ANOVA, F-value=2.397, df=5, p=0.038, **Figure 2.2B**), which decreased during synthetic AP stimulation compared to the period just prior to synthetic AP presentation (Tukey HSD, p=0.019, **Figure 2.2B**, b/c).

Because of the relative change in these two measures, the net influx rate also increased during synthetic AP presentation, relative to presentations of the sponge visual target alone, mineral oil, and just before synthetic AP (ANOVA, F-value=3.657, df=5, p=0.003; d/ e Tukey HSD, SO/AP: p= 0.039, MO/AP: p=0.014, Pre-AP/AP: p= 0.006, **Figure 2.2C**).

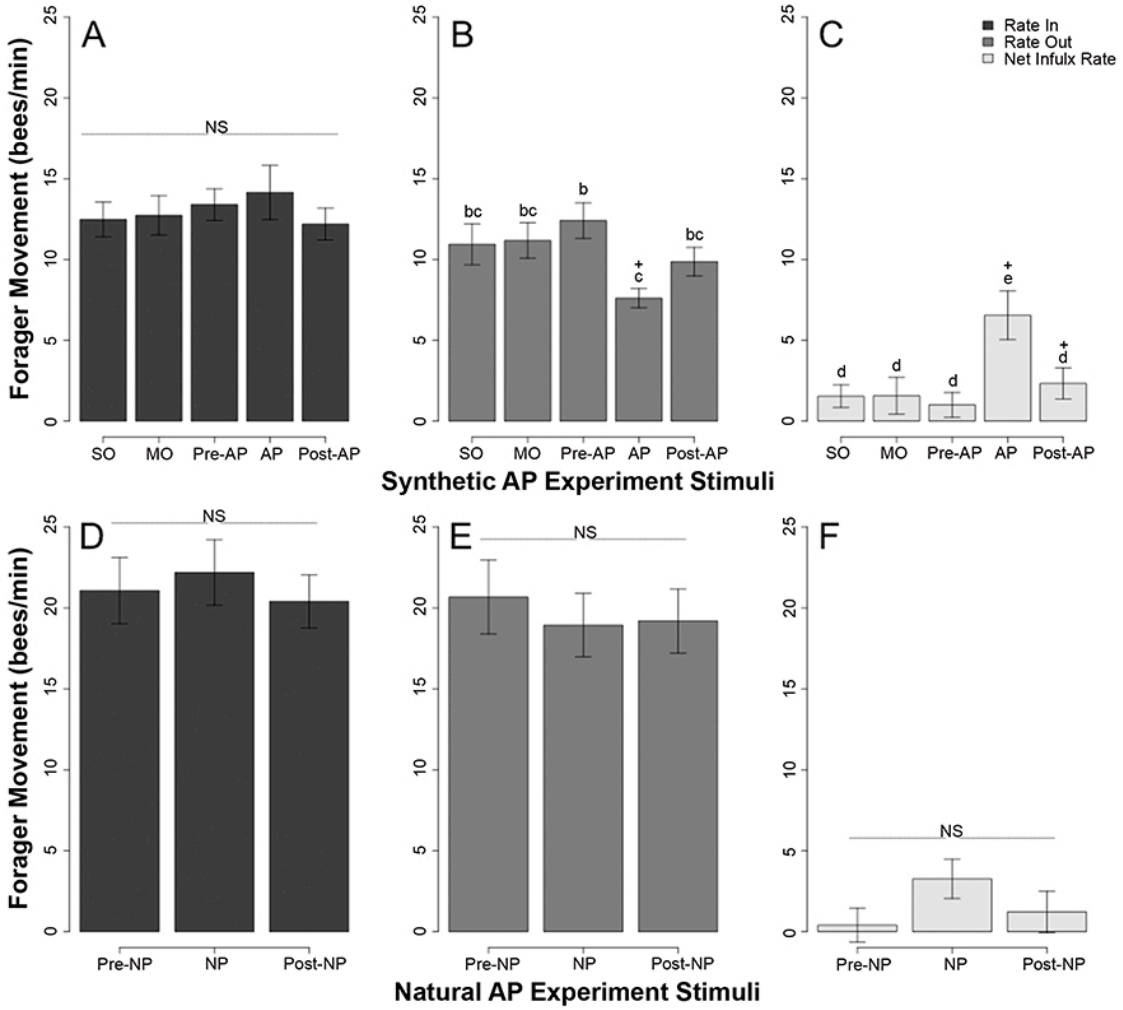


Figure 2.2. The mean movement rates of foragers in bees per min during various stimulus presentations pooled across the synthetic alarm pheromone experiment (**A-C)**. The rate of bees entering the nest (**A,** dark gray); the rate of bees exiting (**B,** gray); and

overall net flux of bees entering the nest (**C,** light gray). Error bars represent standard error of the mean. Treatments are: SO (sponge visual target only), MO (mineral oil on sponge), AP (synthetic alarm pheromone on sponge), Pre- and Post- AP (sponge only before and after synthetic alarm pheromone presentation). Letters denote significant differences: (b) and (c) denote rate out comparisons, and (d) and (e) denote net influx comparisons. (Tukey HSD * $p<0.05$). The mean movement rate of foragers (bees per min) before, during and after presentation of natural alarm pheromone (**D-F)**. The rate of bees entering the nest (**D,** dark gray); the rate of bees exiting (**E,** gray); and overall net flux of bees entering the nest (**F,** light gray). Error bars represent standard error of the mean. Treatments are as follows: NP (crushed bee head), Pre- and Post- NP (sponge only before and after natural alarm pheromone presentation). A total of 342 observations of 9 colonies and 5 types of stimulation for synthetic AP and 100 observations of 9 colonies and 3 types of stimulation for NP.

The same general trend of fewer bees leaving the nest was observed during natural AP simulation, causing the net inflow of bees to be positive. However, the differences were not significant (**Figure 2.2D** rate in: a, ANOVA, F-value= 0.13, df=2, p=0.878; **Figure 2.2E** rate out: b, ANOVA, F-value= 0.154, df=2, p=0.857; **Figure 2.2F** net influx: c, ANOVA, F-value= 0.695, df=2, p=0.502).

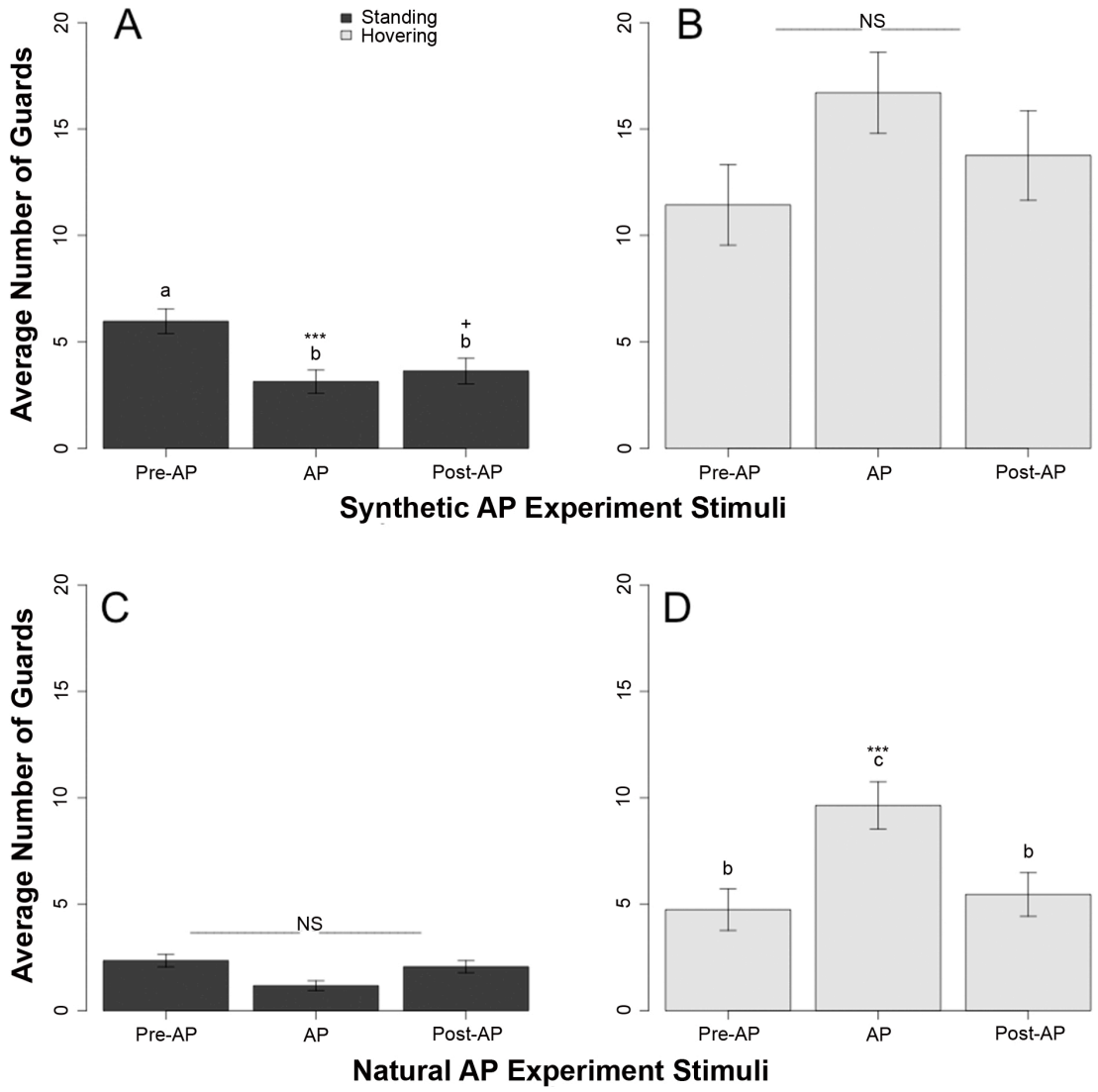


Figure 2.3. The mean number of guards before (Pre-AP), during and after (Post-AP) synthetic alarm pheromone presentation (AP). Standing guards (**A,** dark bars) and hovering guards **(B,** lighter bars). Error bars represent standard error of the mean. Letters denote significant differences: (**a**) and (**b**) show standing guard comparisons, and (**c**) shows hovering guard comparisons. (Tukey HSD *** $p<0.001$, + non-significant, $p=0.053$). The mean number of guards before (Pre-NP), after (Post-NP), and during natural alarm pheromone presentation (NP). Standing guards **(C,** dark bars) and hovering guards

(**D**, lighter bars). Error bars represent standard error from the mean. Letters denote significant differences: (b) and (c) denote hovering guard comparisons (Tukey HSD *** $p<0.001$). A total of 342 observations of 9 colonies and 5 types of stimulation for synthetic AP and 100 observations of 9 colonies and 3 types of stimulation for NP.

Guard Movement: During the presentation of synthetic AP there was a decrease in the number of standing guards (standing: ANOVA, F-value=8.314, df=5, $p<0.001$, Tukey HSD, $p<0.001$; **Figure 2.3A**, a/b), which persisted for several minutes after the removal of synthetic AP (Tukey HSD, $p=0.053$; **Figure 2.3A**, a/b+). However, the change was not significant for hovering guards (ANOVA, F-value=0.478, df=5, $p=0.792$; **Figure 2.3B**). The same general trends were found for the natural AP, but changes were only significant for hovering guards (**Figure 2.3D**, ANOVA, F-value=15.30, df=2, $p<0.001$; Tukey HSD, $p<0.001$; **Figure 2.3D**, b/c) and not standing guards (ANOVA, F-value= 1.546, df=2, $p=0.219$; **Figure 2.3C**). The differences in behavior toward natural and synthetic AP presentations could have been due to odor intensity differences between a single crushed *T. angustula* head and the 1% AP mixture used for synthetic stimulation. In addition, other stimuli may have been present in the crushed head.

There was a significant change in flight behavior in the presence of both synthetic and natural AP stimuli. The bees, presumably hovering guards, would perform an erratic flight pattern in which they would quickly fly back and forth in large loops (approximately 12 cm in each direction) within an approximately 0.5 meter radius around

the nest. This was apparent in an increase in the overall activity and flight velocity of bees around the nest (See supplementary video).

We quantified the overall activity of colonies during different stimulus presentations by tracking frame-to-frame pixel changes (see Methods; Birgiolas et al., 2017). There was significantly more activity during AP stimulation compared to mineral oil only (MO) stimulation for 5 of the 6 initial AP exposures (paired t-tests, $p<0.001$, 6^{th} at $p=0.19$). AP activity was also significantly higher for 27 of 28 paired observations when including all observations irrespective of stimulation timing (paired t-test, $p<0.001$).

Based upon the above results, we felt the responses to synthetic and natural AP were similar enough to justify exclusive use of synthetic AP for further experimental manipulations. Use of synthetic AP controls for the consistency of stimulation intensity and also for other potentially confounding volatile chemicals that may be present in the heads of *T. angustula* guards.

Non-associative plasticity of alarm responses

We binned our data by elapsed time since previous AP exposure in order to observe the effects of repeated alarm stimulation on colony defensive behavior of *T. angustula*. We found evidence for a habituation-like effect on both guard and forager behavior. This change in behavior was not an effect of circadian rhythms, because there was no effect of time of day on the forager movement rates or colony activity within the windows we

observed. However guard numbers varied throughout the day as previously described by Grüter et al. (2011) (**Figure 2.S3**).

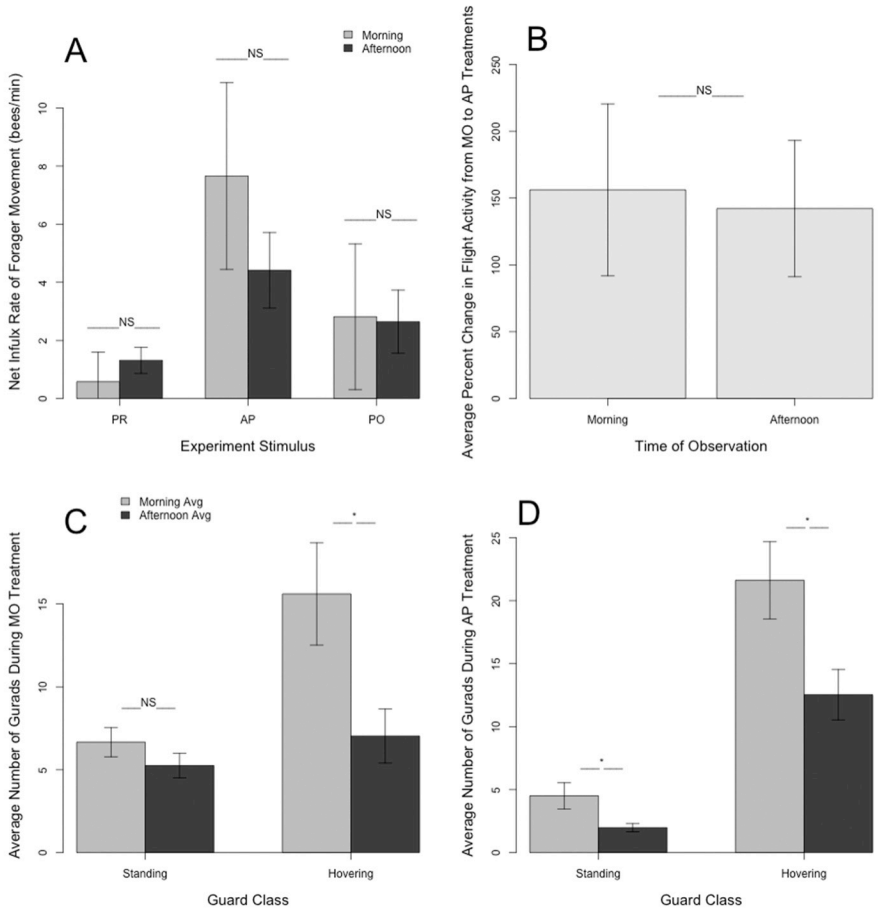


Figure 2.S3. **A**. The mean net inflow of bees before (PR), during (AP) and after (PO) synthetic alarm pheromone presentation pooled by time of day. All morning presentations are denoted in light gray and all afternoon presentations are denoted in dark gray (PR: ANOVA, F-value=0.103, df=1, p=0.05, AP: ANOVA, F-value=1.01, df=1, p=0.32, PO: ANOVA, F-value=0.004, df=5, p=0.948). Error bars represent standard error of the mean. Analysis of a total of 212 observations of 6 colonies, and 3 periods of stimulation before during and after AP. **B**. The mean percent change in flight activity (measured by

frame-to-frame changes in video pixels) from mineral oil (MO) to synthetic alarm pheromone presentation (AP) presentation pooled by time of day presented (ANOVA, F-value=0.483, df=1, p=0.495). Error bars represent standard error of the mean. Analysis of 55 observations of 6 colonies. **C**. The mean number of guards during mineral oil (MO) presentation pooled by time of day. Column shading as in 'A'. No effect of time of day on the number of standing guards (F-value=1.47, df=1, p=0.2330) but a significant effect of time as reported by Grüter et al. (2011) (ANOVA, F-value=5.84, df=1, p=0.02). Error bars represent standard error of the mean (Tukey HSD *p<0.05). Analysis of 37 observations of 6 colonies during MO stimulation. **D**. The mean number of guards during synthetic alarm pheromone (AP) presentation pooled by time of day. Column shading as in 'A'. During AP there was a significant effect of time of day on both the number of standing (ANOVA, F-value=6.158, df=1, p=0.0180) and hovering guards (ANOVA, F-value=5.494, df=1, p=0.015). Error bars represent standard error of the mean (Tukey HSD *p<0.05). Analysis of 37 observations of 6 colonies during AP stimulation.

First we analyzed attacks on either the sponge or the filter paper containing AP using the two measures described in Table 1. There was a significant effect of stimulation interval (SI) on the number of bees attacking and a non-significant effect on the number of attacks counted (**Figure 2.4A**, number of bees attacking: ANOVA, F-value=8.097, df=5, p=0.002, **Figure 2.S5A**, **Figure 2.4B** number of attacks: ANOVA, F-value=2.766, df=5, p=0.08; **Figure 2.S5B**). By both measures, defensive behavior was reduced for AP stimulation 2 hrs after presentation (Tukey HSDs; **Figure 2.4A** number of bees attacking:

first vs. <2 hrs, p=0.009; **Figure 2.4B** number of attacks: first vs. <2hrs, p=0.055). The reduction persisted for up to 12 hrs (Tukey HSDs, first vs. 2-12 hrs, p=0.039; <2 vs. 24-48 hrs, p=0.191; <2 vs. >48 hrs, p= 0.089; **Figure 2.4A**). Defensive behavior returned to initial levels at 12 or more hrs after exposure (Tukey HSDs, first vs. 2-12 hrs, p=0.246; **Figure 1.4A**,**B**, **Figure 2.S5A,B**).

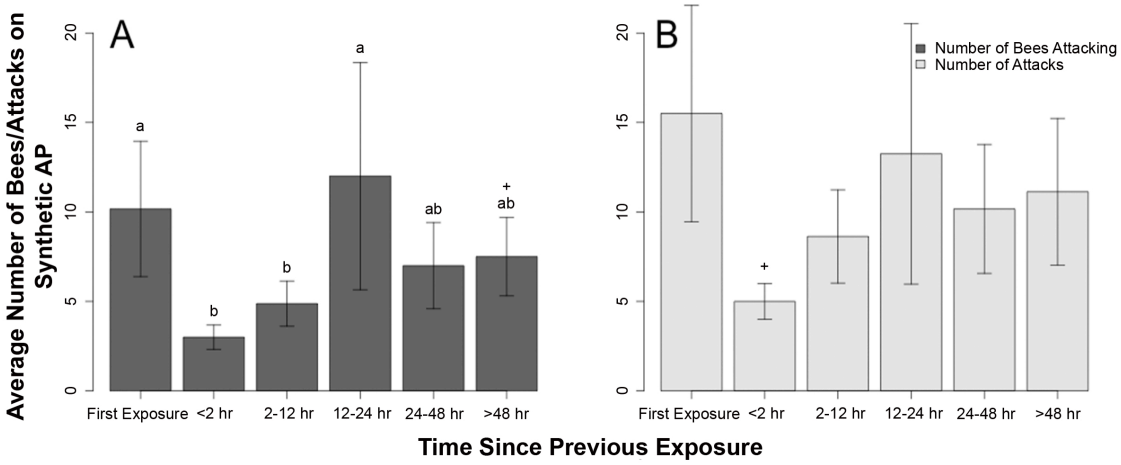


Figure 2.4. The mean number of attacks and estimate of bees attacking during synthetic alarm pheromone presentations binned by time since previous exposure to synthetic alarm pheromone. The number of bees attacking (**A**, dark gray), and the number of attacks on synthetic alarm pheromone stimulus (**B**, light gray). Error bars represent standard error of the mean. Letters denote significant comparisons: (a) and (b) denotes number of bee estimate comparisons, and (c) denotes number of attack comparisons (Tukey HSD *p<0.05, **p<0.01, + non-significant p= 0.055). A summary of 39 alarm presentation observations of 6 colonies.

We next analyzed the impact of the time since AP stimulation on the net rate of foraging activity, in the same manner as above, during both AP and mineral oil stimulation (**Figure 2.5**, **Figure 2.S5C**). We found a significant effect of the time since previous exposure on movement rate both during AP presentation (**Figure 2.5A** rate in: ANOVA, F-value=8.249, df=5, p=0.003, **Figure 2.5B** rate out: ANOVA, F-value=8.887, df=5, p=0.002, **Figure 2.5C** net influx: ANOVA, F-value=8.249, df=5, p=0.002; **Figure 2.S5C**) and during mineral oil presentation (**Figure 2.5D** rate in: ANOVA, F-value=5.362, df=5, p=0.012; **Figure 2.5E** rate out: ANOVA, F-value=3.496, df=5, p=0.043; **Figure 2.5F** net influx rate: ANOVA, F-value= 6.839, df=5, p=0.005). Foragers displayed reduced rates of nest entry 2-12 hrs after AP exposure but not at the shortest simulation interval during both AP (Tukey HSD, rate in: first vs. 2-12 hr, p=0.001, <2h vs. 2-12 hr, p=0.003; Fig 2.5A-C, **Figure 2.S5C**) and mineral oil stimulation (Tukey HSD, first vs. 2-12 hr, p=0.009; <2 vs 2-12 hr, p=0.062; **Figure 2.5D-F**).

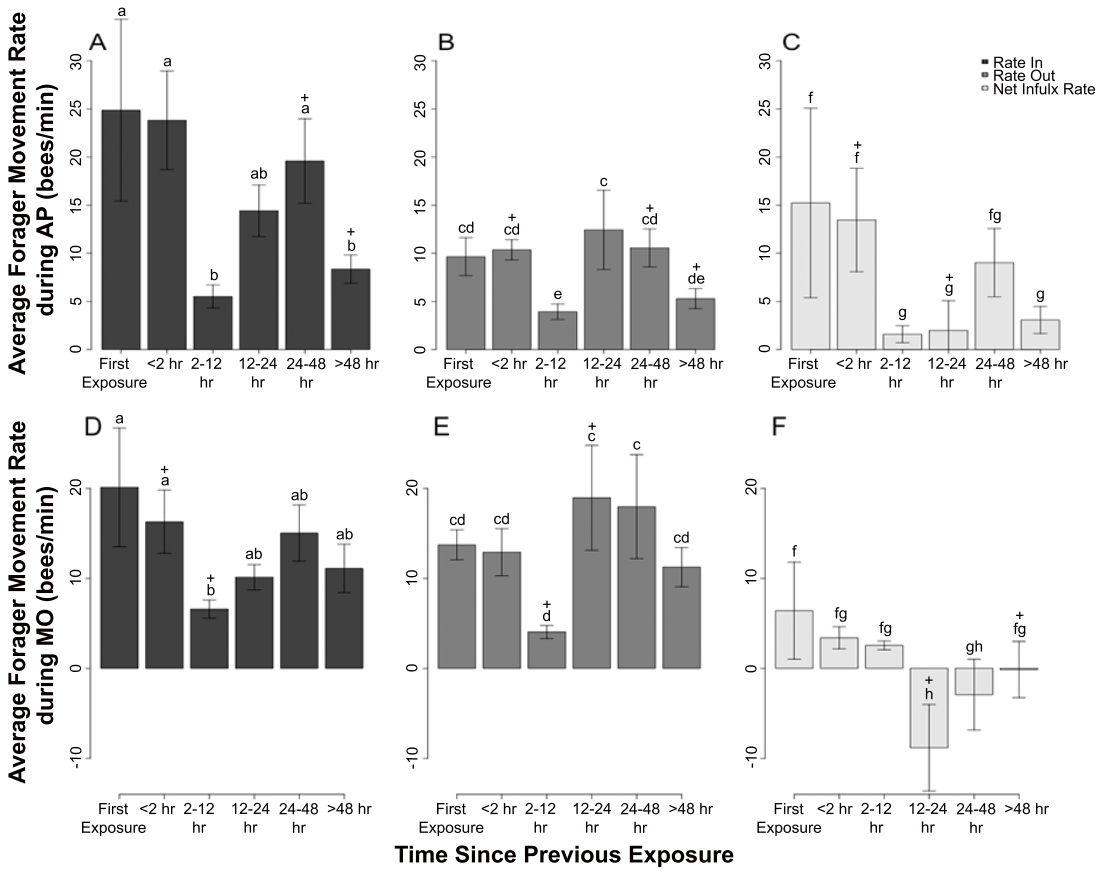


Figure 2.5. **A-C.** The mean forager movement rate (bees/min) during synthetic alarm pheromone presentation binned by time since previous exposure to synthetic alarm pheromone. Rate of bees entering the nest (**A**, rate in, dark gray), rate of bees exiting the nest (**B**, rate out, medium gray), and net rate of bees entering the nest (**C**, net, light gray). Error bars represent standard error of the mean. Letters denote significant comparisons: (a) and (b) denote rate in comparisons, (d), (c), and (e) denote rate out comparisons, and (f) and (g) denote net influx rate comparisons. (Tukey HSD + non-significant, $0.067 < p > 0.05$) **D-F**. The mean forager movement rate (bees/min) during mineral oil only presentation binned by time since previous exposure to synthetic alarm pheromone. Rate of bees entering the nest (**D**, rate in, dark gray), rate of bees exiting the nest (**E**, rate out,

medium gray), and net rate of bees entering the nest (**F**, net, light gray). Error bars represent standard error of the mean. Letters denote significant comparisons: (a) and (b) denote rate in comparisons, (d) and (c) denote rate out comparisons, and (f), (g) and (h) denote net influx rate comparisons. A summary of 39 alarm presentation observations of 6 colonies.

During AP stimulation, colonies also showed decreased rates of bees exiting the nest 2-12 hrs after previous exposure (Tukey HSD, rate out: first vs. 2-12 hr, $p=0.03$, <2 vs. 2-12 hr, $p=0.017$; **Figure 2.5B**). This corresponds to the first experiment presented (**Figure 2.1A-B**), in which the rate of bees exiting seemed to be most affected by the presence of AP (**Figure 2.2B**). Interestingly, during mineral oil stimulation bees also showed reduced exit rates compared with longer SI times (Tukey HSD, 2-12 hr vs. 12-24 hr, $p=0.067$; 2-12 hr vs. 24-48 hr, $p=0.049$; **Figure 2.5E**), but not between the first exposure and subsequent rates during the shortest interval.

When we measure the net forager activity during AP stimulation, the net influx rate began positive; then with repeated AP stimulation it reduced to a value similar to that observed during mineral oil stimulation. The next influx rate became closer to a zero value but did not fully recover within the shorter stimulus intervals (first vs. 2-12 hr, $p=0.007$, <2 vs. 2-12 hr, $p=0.017$; **Figure 2.5C**). During mineral oil exposure we also observed differences in net influx rates (**Figure 2.5F**). Strangely, the net influx rate was significantly more reduced at stimulus intervals greater than 12 hrs (Tukey HSDs, first

vs. 12-24 hrs, p=0.004; <2 vs. 12-24 hrs, p=0.018, 2-12 vs. 12-24 hrs, p=0.018; **Figure 2.5F**) and normalized at stimulus intervals greater than 48 hrs. Given the previous observation that mineral oil and the presence of visual targets did not have an effect on forager activity, this suggests a more long-term alteration in forager activity after several AP stimulations.

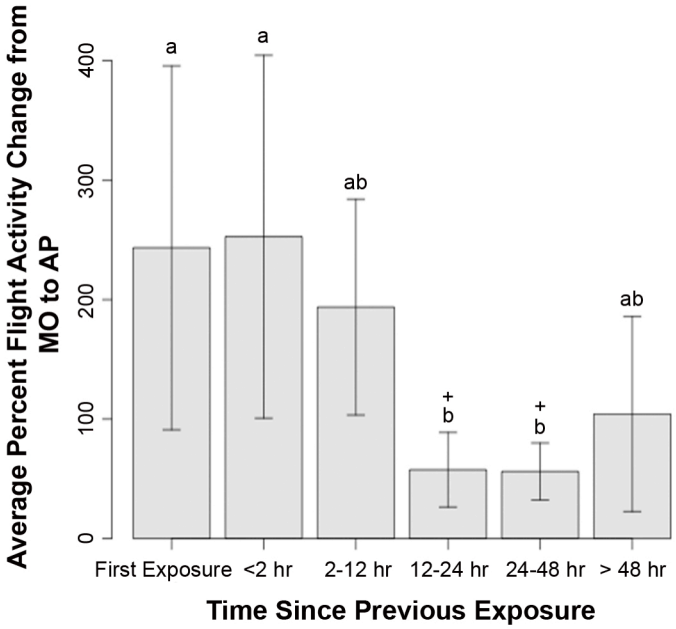


Figure 2.6. The mean percent change in flight activity from synthetic alarm pheromone (AP) and mineral oil (MO) presentations (measured by frame-to-frame changes in video pixels) binned by time since previous exposure to AP. Error bars represent standard error from the mean. The letters denote comparisons with (b+) as approaching significance—p=0.1—from (a) and no difference from (ab). A summary of 39 alarm presentation observations of 6 colonies

Last, we looked at the impact of the rate of AP stimulation on overall activity. We found there was an effect that approached but did not reach statistical significance of repeated AP stimulation on the overall activity at the nest entrance (ANOVA F-value=10.47, df=5, p=0.089; **Figure 2.6**, **Figure 2.S5D**).

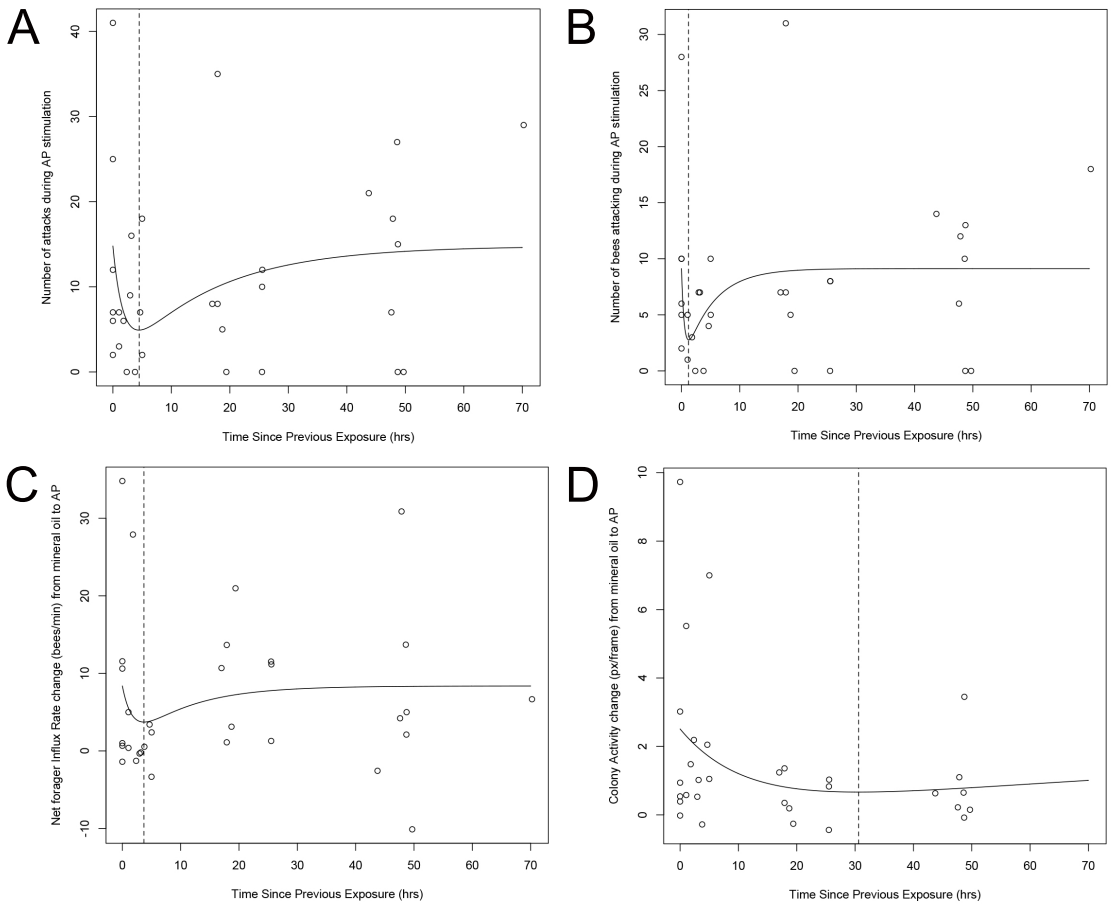


Figure 2.S5. Plots of measures during alarm pheromone (AP) exposure minus the measure during mineral oil exposure versus the time (t) since previous AP exposure (hrs). The points in each plot are the data and the solid line is a model fit of the data, described in supplemental analyses. The vertical dashed line indicates the saddle or inflection point as defined by the model fit, and separates the decay phase and the recovery phases

observed in the data. **A.** The number of attacks during AP stimulation, this is also the difference between this measure and mineral oil, as there were never any attacks observed during mineral oil stimulation. The model fits the saddle point to be 4.5 hrs. The decay phase shows a non-significant effect of t (GLM, estimate= 0.535, z-value= 1.704, p= 0.089), a significant effect of the number of AP exposures (GLM, estimate= -1.539, z-value= -3.766, $p < 0.001$), and a non-significant interaction (GLM, estimate= -0.032, z-value= -0.201, p= 0.841). The decay model still has a better fit of the data than the null (χ^2, p=0.02). The recovery phase shows a non-significant effect of t (GLM, estimate= -0.01, z-value= -0.835, p= 0.404), a significant effect of number of AP exposures (GLM, estimate= -0.662, z-value= -4.483, $p<0.001$), and a significant interaction (GLM, estimate=0.009, z-value= 2.527, p= 0.011). The recovery model fits significantly better than the null (χ^2, $p < 0.001$). **B.** The number of bees attacking during AP stimulation, this is also the difference between this measure and mineral oil, as there were never any attacks observed during mineral oil stimulation. The model fits the saddle to be 1.2 hrs. The decay phase shows a significant effect of t (GLM, estimate=-1.162, z-value= -2.853, p= 0.004). There were not enough measures in the 1.2 hrs to test the number of exposures or the interaction. The recovery phase shows a significant effect of t (GLM, estimate=0.028, z-value= 2.245, p= 0.025), a non-significant effect of number of AP exposures (GLM, estimate= -0.242, z-value= -1.609, p= 0.12), and a non-significant interaction (GLM, estimate= -0.001, z-value= -0.533, p= 0.594). The recovery model is significantly better fit than the null (χ^2, $p<0.001$). **C.** The net forager influx rate (bees/min) difference between AP and mineral oil stimulation plotted against the time

since previous alarm pheromone exposure. The model fits the saddle to be at 3.7 hrs. The decay phase shows non-significant effect of both t (GLM, estimate=5.27, t-value= 0.399, p=0.701) and number of AP exposures on observed measures (GLM, estimate=-0.466, t-value=-0.03, p=0.977), and no difference from null model (χ^2, p=0.9). However, the recovery phase shows a significant effect of t (GLM, estimate=1.159, t-value=3.426, p=0.003), number of exposures (GLM, estimate=7.935, t-value=2.198, p=0.04), and an interaction (GLM, estimate=-0.283, t-value=-3.204, p=0.005). The recovery has a significantly better fit than the null model (χ^2, p=0.003). **D.** The colony activity change during AP simulation and mineral oil stimulation versus the time since previous AP exposure (hrs). The model fits the saddle point to be 30.6 hrs. Both the recovery and the decay showed non-significant differences from the null models (χ^2, p >0.6) and no significant effect of either t or number of AP exposures on activity (GLM, p>0.5). There was also no significant interaction between the two (GLM, p >0.6)

Associative alarm responses

We also attempted to pair AP as an unconditioned stimulus with two different non-pheromone/kairomone odor conditioned stimuli (CSs). We compared the first response to the CS with subsequent responses after several CS-AP (US) pairings. There was no impact of experience (CS-US pairings) on response to the CS for the net influx rate (ANOVA, F-value=2.332, df=1, p=0.129; **Figure 2.S4**). Guard measures and nest activity measurements were also not significantly different (**Table 2.S1**).

Table 2.S1. The test statistics for ANOVA and paired t-test outputs of the novel odor-alarm pheromone paring tests. Presenting for number of bees attacking, number of attacks, and average percent flight activity change between mineral oil and alarm stimulation. All tests are non-significant.

ANOVA	F-Value	D.F.	P-value
Number of bees Attacking	1.216	1	0.272
Number of Attacks	0.967	1	0.327
Two-Tailed T-Test	**T-Value**	**D.F.**	**P-value**
Average Percent Flight Activity Change	0.417	20	0.681

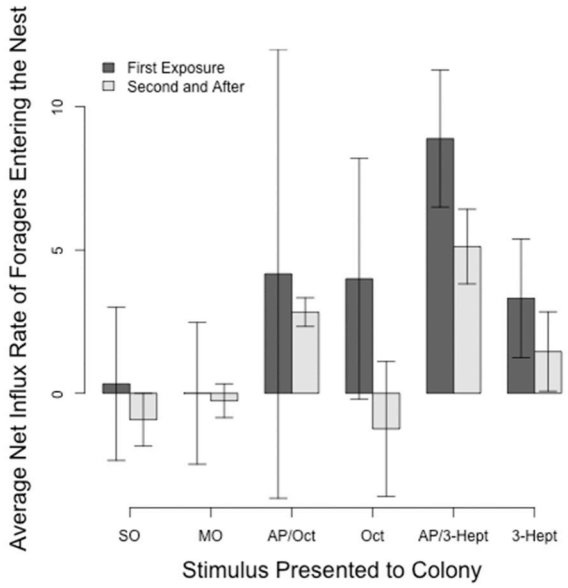


Figure 2.S4. The mean net influx rate of bees entering the nest (bees/min) during various stimulus presentations and pairings: sponge only present (SO), sponge with mineral oil

stimulus (MO), sponge with synthetic alarm pheromone and Octane pairing (AP/Oct), sponge with octane only (Oct), sponge with synthetic alarm pheromone and 3-Heptanal pairing (AP/3-Hept), and sponge with 3-Heptanal only (3-Hept). First exposure to stimulus is denoted in dark gray and the pooled following stimulus presentations are denoted in light gray. Error bars represent standard error of the mean. We are only presenting the net influx rates as those seemed to be the some of the most robust measures we tested to determine significant impacts on colony behavior. Summary of 163 observations of 5 colonies.

DISCUSSION

Adaptive topology of colony defense behavior

The defensive response of *Tetragonisca angustula* involves both 'standing' and 'hovering' guards (C Grüter et al., 2011; Christoph Grüter, Segers, Santos, et al., 2017; D Wittmann, 1985). The former take up positions on the nest entrance tube either just inside the entrance or around the outside of the tube near the entrance. Hovering guards fly in a fairly stable position relative to the nest entrance tube and are oriented toward the entrance (Kelber & Zeil, 1990; Zeil & Wittmann, 1989). We find that during presentation of synthetic or natural alarm pheromone the number of hovering guards increases. The increase most likely results from the recruitment of the standing guards, because the number of outbound departures from the nest entrance is reduced. However, the increase in total hovering guards is on average greater than the number reduced from the standing pool (**Figure 2.S2**). This would suggest that there was recruitment of guards from inside

the nest or incorporation of returning foraging bees as exterior nest guards. Furthermore, we find that the colony-level defensive response changes with experience, and these changes are most consistent with habituation.

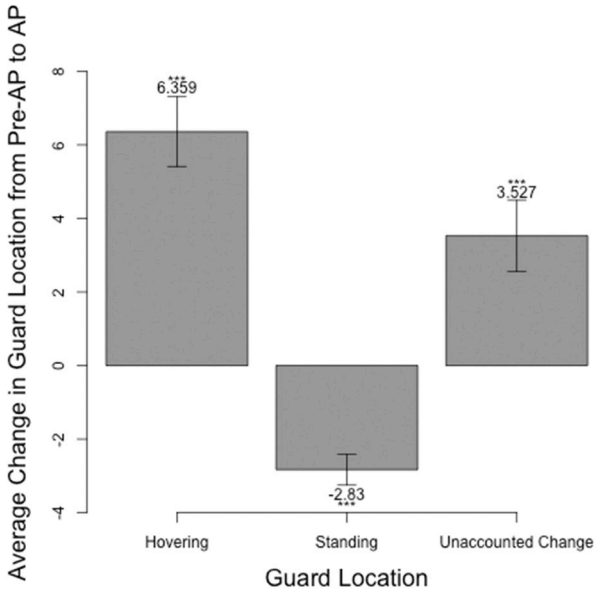


Figure 2.S2. The mean change in guard number from just before (pre-AP) to during synthetic alarm pheromone presentation (AP). There is also an unaccounted change in total guard number from before to after AP presentation, represented by the unaccounted change column. Error bars represent standard error from the mean. (two-tailed, one sample students t-test ***$p<0.001$) Standing: two tailed t-test, t-value=-6.81, df=32, $p<<<0.001$; hovering: t-test, t-value=6.69, df=32, $p<<<0.001$, unaccounted difference: t-test, t-value= 3.648, df=32, $p<0.001$). Analysis of a total of 34 observation periods during AP stimulation of 6 colonies.

One of the main threats to any stingless honey bee colony comes from other meliponine bee species that raid colonies (Cunningham, Hereward, Heard, De Barro, & West, 2014; Christoph Grüter, Segers, Menezes, et al., 2017; Sakagami et al., 1993; Von Zuben et al., 2016). In the context of a non-nestmate or heterospecific scout discovering the nest entrance, the goals of a colony should be as follows. Outside the nest, guards need to quickly immobilize the threat and minimize loss. In order to accomplish this, guards may actively search for and eliminate foreign (non-nestmate and heterospecific) scouts outside the nest. Inside the nest, nestmates should maintain the integrity of colony brood and other critical resources in the event that any raiders gain entry.

The responses we observed during alarm stimulus presentations are consistent with this interpretation. The colony increases the number of actively hovering guards. At the same time, outgoing activity is reduced, which increases the number of bees within the nest who could defend the colony and the queen if intruders breach the perimeter. Other reports have described that, in the event of a raid and loss of the nest entrance integrity, nestmates stand over brood and other critical resources to reduce the potential impact a raid event has on the colony survival (Sakagami et al., 1993).

Qualitatively the responses to both the natural and synthetic alarm pheromone stimuli are very similar. However, there were clear quantitative differences between the natural and synthetic alarm pheromone stimuli response levels. These differences were most likely due to two possibilities. First there could be quantitative differences in alarm substance

released between a single crushed *T. angustula* head and the 1% AP mixture we used for synthetic stimulation. Second, there may have been other volatiles in the bee head, which have not been completely quantified or tested in *Tetragonisca angustula*. It is clear from the work of Wittmann et al. (1990) that the responses of *T. angustula* to their alarm components are robust to chemical composition.

The responses we see to both natural and synthetic alarm phermone stimulation are consistent with those observed in prior *T. angustula* studies as well as other studies involving social bees. A recent study by Gong et al. (2017) has shown reduced forager activity in response to the primary component of the honeybee alarm pheromone, isopentyl acetate, in both the Asian and European honey bees. This is consistent with what we observe here with *T. angustula* foragers. Additionally, reduced forager responsiveness has also been demonstrated in the lab using harnessed bees, which perform very poorly in proboscis extension tasks in the presence of alarm substance (Urlacher et al., 2010). Thus the natural and synthetic alarm pheromone responses we observe in *T. angustula* appear to be robust across social bees. However, the unique guard behaviors of *T. angustula* provide additional insight into the whole colony response, which is not possible to easily observe in other bee species.

Non-associative modification of the defense response

The colony-level defense behaviors of *T. angustula* are modified by experience in ways that are consistent with habituation. Habituation is an adaptive behavioral phenomenon

through which the response to a stimulus decreases with repeated exposure (Harris, 1943; Rankin et al., 2009; Rescorla, 1988; Thompson & Spencer, 1966). Thomson and Spencer (1966) describe several parametric characteristics of behavioral habituation, later revised by Rankin et al. (2009), which can be used to dissociate it from sensory adaptation. We report two critical points for demonstrating habituation. The first criterion is a decrease in behavioral response with repeated stimulation. We find a decrease in guard attacks, forager movement, and activity in response to repeated alarm pheromone stimulation. Second, we observe spontaneous recovery to approximately normal baseline defensive responses with intervals of 24 to 48 hrs.

Our experiments cannot fully eliminate sensory adaptation, which refers to a decline in response of olfactory sensory cells that detect a stimulus, such as alarm pheromone. Sensory adaptation is well known to occur in olfactory receptor neurons, and brief exposure to an odor produces both a decline in responsiveness of the sensory neuron as well as a decline in behavioral response to the odor (Colbert & Bargmann, 1995; Kaissling, Strausfeld, & Rumbo, 1987; Kurahashi & Menini, 1997). However, because of the time frames involved in our tests, sensory adaptation is less likely. The longest lasting form of sensory adaptation affects sensory cells on the order of at most a few hrs (Colbert & Bargmann, 1995; Zufall, Leinders-Zufall, & Greer, 2000). Reported sensory adaptation durations vary by animal: salamanders 6.5 mins (Zufall et al., 2000); the fruit fly for 1.5 min (Störtkuhl, Hovemann, & Carlson, 1999); house fly for 15 mins (Kelling, Ialenti, & Den Otter, 2002); the worm *C. elegans* for approximately 3 hrs (Colbert & Bargmann,

1995); silkworm moth for approximately 1 hr in isolated antennae (Kaissling et al., 1987); the rabbit for 30-50 min when adapted for 1 hr (Chaput & Panhuber, 1982). In our study we find reduced behavioral responses to olfactory alarm stimulation lasting up to 12 hrs, which would be unlikely to be due to sensory adaptation.

Therefore, reductions in behavior that we report could be due to habituation, which can last at least for days as it progresses through different forms of memory consolidation (Menzel, 1999; Tully, Preat, Boynton, & Del Vecchio, 1994). In honeybees long-term appetitive habituation has been shown to last at least 24 hrs (Bicker & Hähnlein, 1994). We did not, however, have the opportunity to test additional behavioral characteristics that affect habituation, such as: stimulus strength; generalization of habituation to other stimuli; or dishabituation. The last characteristic refers to re-establishment of the behavioral response just after presentation of a strong sensitizing stimulus, such as disruption or a disturbance of the colony, or perhaps presentation of the alarm pheromone of a parasitic species of stingless honey bee (e.g. *Lestrimelita*; Sakagami et al., 1993). Demonstration of dishabituation, coupled with electrophysiological measurements from the sensory cells on the antennae, would be the next essential steps in testing the habituation hypothesis for *T. angustula*.

Circadian rhythm cannot account for the reduction in behavior that we observed. Five colonies were initiated in the morning and four others were initiated several hours later. As a result, the alarm behaviors as a function of initial treatments were tested at different

times of day. Furthermore, the behaviors that change as a result of experimental manipulation did not show differences between the two onset times. We observed circadian patterns in guard number as had previously been shown (C Grüter et al., 2011), but that would not account for the reductions we report.

At first it may seem puzzling that a colony would reduce alarm responses to repeated alarm pheromone delivery. A colony needs to be able to maintain the defense of resources especially in the face of repeated attack. The decline in responsiveness we report could allow a colony to 'tune' the alarm response in a way that is adaptive to different levels of regularly occurring threats. A low level of threat might consist of an occasional scout bee from a potential raiding colony. This situation could be analogous to the condition that we presented to the colonies. Bees generally did not die during alarm presentations (with the exception of the few bees that performed a 'death grip' on the AP filter paper), and there were no large-scale raids from non-nestmate bees. Thus there was no immediate threat to the colony. In this case, the adaptive response to repeated low-level threats would be to conserve energy and resources by reducing the response to a mild threat. Accordingly, (Tan et al., 2016) have shown colony-level response tuning to wasps of varying threat levels in the Asian honey bee, *Apis cerana*.

We also report that different behaviors show habituation to AP in different time frames. These behaviors represent different behavioral castes within the stingless honey bee colony (Hammel et al., 2016). Attacking guards habituate most rapidly, followed by

foragers at between 2-12 hrs, and then general activity reduces during from 12 to 48 hrs after stimulation. These responses provide an adaptive response that could reduce disruption of foraging activity and minimize guard energy expenditure, while still balancing the 'vigilance' of the colony response to a threat.

Long term responses to repeated alarm pheromone stimulation

Colony responses, including both forager activity and guard behavior, adapt to repeated alarm stimulation for extended periods of time. Early comparisons establish that the presence of mineral oil and the visual target do not have an impact on forager activity. Yet, we see changes in forager activity after repeated alarm pheromone (AP) stimulation during the mineral oil observation period (**Figure 2.5D-F**). This suggests that repeated alarm pheromone stimulation has impacts on foraging activity for at least for 2 to 24 hrs after the window of AP observation. Moreover, the reduction generalizes to the tests with mineral oil, indicating that it is a broad, colony-wide response. We also observe long term changes in guard behavior but they only manifest during alarm presence. The altered response profiles for both foragers and guards are long lasting (2-48 hrs), but manifested in different ways. Guards only attack or demonstrate activity during AP stimulation, and therefore only demonstrate altered response profiles during AP stimulation (**Figure 2.4**). Foragers on the other hand are always active, and thus manifest altered activity with and without AP stimulation for 2-12 hrs after AP stimulation but without alarm pheromone present (**Figure 2.5**).

Associative conditioning with alarm pheromone

We found no evidence that colonies of *T. angustula* modified their behavior in response to novel odors paired with synthetic AP. Thus we cannot at present conclude that the association of an AP with kairomones of other bees can modify the colony response to the kairomone. However, we occasionally observed individuals performing 'death grip' behaviors on filter paper with novel odor in addition to the filter paper containing AP. The most parsimonious explanation is that the bees stimulated by the AP were simply performing the 'death grip' on any novel stimulus in the vicinity of the AP stimulus. Or the biting behavior could be directed at any odor near the AP. It remains possible, however, that the bees could have learned the association of AP with the novel odor and performed the 'death grip' in response to the novel odor, just as they would to the AP. Recent work by (Dawson, Chittka, & Leadbeater, 2016) reports individual associative learning using alarm pheromone paired with colored light in the honeybee, *Apis mellifera*. So it is possible that conditioning at the individual-level occurred, but we were unable to capture this with our colony-level measures.

Group versus individual conditioning

All of our experiments involved treating groups of individuals. We therefore report evidence of adaptive group-level modification of behavior. This could be acquired in two ways. First, it could be acquired through individual experience. Second, it could be acquired through indirect experience via colony communication or modification of some colony level signal. We assume that a decline in response when tested several hrs later

reflects, in some way, individual learning via one or both means. However, we did not track individuals in our experiments, which would have been difficult because of the situation of the colonies and because of the size of the bees. Therefore, we have no way of knowing if the individuals present during the test were the same ones present during the treatment. It therefore remains an open, interesting question how individuals learned in our experiments. According to Grüter et al. (2011), individual standing guards perform guarding behavior approximately 1-2 hrs at a time, and hovering guards perform guarding behavior for about 1 hr at a time in *T. angustula*. It is not currently known how frequently a *T. angustula* guard performs guarding behavior. Individual honeybees (*Apis mellifera)* perform both guard and foraging tasks with some circadian regularity (Moore, Angel, Cheeseman, Fahrbach, & Robinson, 1998; Troen, Dubrovsky, Tamir, & Bloch, 2008), thus it is possible that *T. angustula* switches between these activities. However, given that the reduction of attacking behavior and foraging activity extends beyond the 2 hr window of initial testing, it seems likely that different individual bees were involved between initial exposure and testing at different time periods up to 12 hrs later. If the individuals differ during a test, then it remains an open question how they learned about the alarm pheromone exposure without having performed the behavior during the initial treatment. In conclusion, our experiments raise the important issue of how individual and colony factors come into play to disseminate learning in groups to shape colony-level defense.

CHAPTER 3

EXPERIENCE-DEPENDENT DEVELOPMENT OF EARLY OLFACTORY PROCESSING IN THE HONEY BEE BRAIN

Abstract

Experience plays a critical role in shaping the central nervous systems of animals, and this plasticity allows animals to adaptively change their responses to stimuli over time. Therefore, it is important to understand the different time frames and mechanisms over which plasticity works. In this study, we explore how a chronic reduction in post-eclosion olfactory experience shapes olfactory learning and olfactory processing in the antennal lobe network of the honey bee, *Apis mellifera*. We placed genetically uniform and age-matched sets of sister honey bees into two different olfactory conditions. In one condition, we reduced olfactory experience of foraging bees by placing them in a tent in which both sucrose and pollen resources were associated with a single odor, 1-hexanol. In the second condition, bees were allowed to freely forage and receive a diversity of resource-associated olfactory experiences. We measured the antennal lobe glomerular responses to odors using calcium imaging, and we found that reducing the olfactory experience of bees also reduced the natural inter-individual variation in the glomerular response profiles to odors. We next measured the impact of this treatment in a learning assay. We found that bees with a reduced olfactory experience had more difficulty picking an odor out of a mixture, which led them to generalize more (or respond similarly) to different mixture components than bees allowed to freely forage outside of

the tent. We also found that bees with a reduced olfactory experience had higher ongoing structural plasticity in their antennal lobes compared with freely foraging bees, suggesting sensory input post-eclosion is necessary to fully develop the antennal lobes. Together, our data show that olfactory experience can affect both the primary olfactory lobe and the behavioral capacity of honey bees. This study and others highlight the potential impact of individual experience at multiple levels (i.e., behavioral, physiological, developmental) of olfaction.

INTRODUCTION

Odors are encoded in early olfactory processing areas—the olfactory bulb or antennal lobes—in the adult brains of animal species (Hildebrand & Shepherd, 1997). Canonically the activity patterns in these brain regions to different odors are conserved across animals and are species specific (Galizia, Sachse, Rappert, & Menzel, 1999), however it is also known that there is plasticity in the output of these activity patterns modulated by odor experience (Fernandez et al., 2009; Locatelli et al., 2013). We hypothesize that the plasticity in activity patterns observed across animals may in part be shaped by differences in experience animals have had at critical periods throughout development and into their adult lives. To date, the exact role of experience in giving rise to inter-individual variation is still an open question. Generally speaking, the olfactory code results from binding of volatile chemicals to peripheral olfactory receptor neurons (ORNs) that then project, via axon terminals, to glomeruli in the primary olfactory neuropil—the olfactory bulb (OB) in mammals or the antennal lobe (AL) in insects

(Buck & Axel, 1991; Hildebrand & Shepherd, 1997). Activity in glomeruli—spherical, highly synapse-rich areas in the primary olfactory neuropils of the central brain—is then influenced by several different types of excitatory and inhibitory local interneurons as well as by modulatory neurons that signal, among other things, reward (Farooqui et al., 2003; Hammer & Menzel, 1998; Ramaekers et al., 2001; Sachse & Galizia, 2002; Schäfer & Bicker, 1986; Shang et al., 2007). Several Projection Neurons (PNs) then transmit signals to higher brain centers (Abel et al., 2001; Kirschner et al., 2006). Despite this high degree of functional similarity across animals from diverse phylogenetic origins, we still do not fully understand the plasticity in the olfactory bulb or antennal lobe, particularly in regard to apparent inter-individual variation in odor dependent activity and behavioral responses to odors

Individual honey bees differ substantially in their cross-glomerular olfactory code, but the bases to this variation remains poorly understood (Arenas et al., 2012; Brown et al., 2004; Brown et al., 2002; J. Carcaud et al., 2012; Fernandez et al., 2009; Galizia & Kimmerle, 2004; Sachse & Galizia, 2002). Individual honey bees likely have very different olfactory experiences. Foragers freely fly in all directions within a several-mile radius to collect pollen and nectar resources from a diversity of floral sources that produce different perfumes to signal these rewards (Menzel, 1985; Wright & Schiestl, 2009). To date, no one has experimentally investigated whether such differences in individual experiences may influence how the brains of individuals process odors.

The antennal lobe and mushroom body neuropils show altered morphological development correlated with age and experience (Brown et al., 2004; Brown et al., 2002; Coss, Brandon, & Globus, 1980; Fahrbach et al., 2003; Fahrbach et al., 1995; Fahrbach et al., 1998; Farris et al., 2001). Age and foraging experience has been correlated with region-specific volume changes in higher-order brain centers, in which there are larger mushroom body sub-regions and more complex dendritic arbors of visually-associated Kenyon cells within the mushroom body collar (Durst, Eichmüller, & Menzel, 1994; Fahrbach et al., 2003; Fahrbach et al., 1998; Farris et al., 2001). Additionally, foraging experience correlates with changes in the volume and synaptic density of glomerulus 44 within the antennal lobe (Brown et al., 2004; Brown et al., 2002). However, as yet it remains unclear whether experience affects the mechanisms of odor processing.

Within the antennal lobe there are several known factors that drive can drive odor-experience dependent plasticity in olfactory codes. Odor experience can drive short-term plasticity in the physiological responses to odors within an animal via non-associative, latent inhibition, or appetitive odor associations as detected via Ca^{2+} imaging in the antennal lobe (Fernandez et al., 2009; Locatelli et al., 2013). In associative odor learning octopamine (OA) is known to be critical in supporting odor associative conditioning in both fruit flies, *Drosophila melanogaster*, (Burke et al., 2012; Bertram Gerber, Stocker, Tanimura, & Thum, 2009; Schwaerzel et al., 2003) and honey bees (Farooqui et al., 2003; Hammer & Menzel, 1998). One potential candidate receptor that may facilitate this odor-associative plasticity in the honey bee is the octopamine 1 receptor (AmOA1),

which is also known to modulate Ca^{2+} levels within cells (Grohmann et al., 2003). Previous work localizing the AmOA1 receptor in the honey bee antennal lobe indicated that this receptor is expressed in inhibitory local neurons (LNs) (I. Sinakevitch et al., 2011; I. T. Sinakevitch et al., 2013). Sinakevitch et al. (2011) also described inter-individual variability in antennal lobe AmOA1 expression patterns. These local neurons are responsible for modulation of the olfactory code in the antennal lobe and synapse onto both projection neurons and other local neurons (Fernandez et al., 2009; Locatelli et al., 2013; Sachse & Galizia, 2002). Additionally, the odor response profile in the projection neurons of the antennal lobe is altered as a result of associative conditioning in a manner that appears to enhance odor discrimination (Fernandez et al., 2009; Locatelli et al., 2016; Locatelli et al., 2013; Rath, Giovanni Galizia, & Szyszka, 2011). This work has led to the hypothesis that octopamine could modulate this process via AmOA1 and local neuron activity.

In this work we, for the first time, control the odors foraging honey bees have access to associated with their collected resources and then directly measure the inter-individual plasticity at three levels: responses of projection neurons in the antennal lobe, odor mixture learning and recall, and expression of proteins in the antennal lobe correlated with neural plasticity and odor associative learning. We hypothesize that the unique, individual associative odor experience drives much of the inter-individual plasticity that has been observed across animals.

RESULTS

Honey bee olfactory system and AmOA1 expression with development

The honey bee olfactory system is comprised of the antennae, the antennal nerve (AN), the antennal lobe (AL), projection neuron (PN) tracks, and higher-order olfactory centers (**Figure 3.1A)**. The primary olfactory center in the central brain is the AL, which consists of ~170 glomeruli and an aglomerular neuropil (**Figure 3.1B**) (Robertson & Wanner, 2006; I. T. Sinakevitch et al., 2017). The glomeruli are divided into a cortex (or the outer region of the glomeruli) and a core region (Galizia & Sachse, 2010; Hildebrand & Shepherd, 1997; Nishino et al., 2009). The cortex region of the glomeruli contains synapses, which primarily receive direct inputs from the axon terminals of olfactory receptor neurons (ORNs) via the AN (Nishino et al., 2009). The core region contains synapses primarily comprised of local interneurons (LN) and projection interneurons (PN).

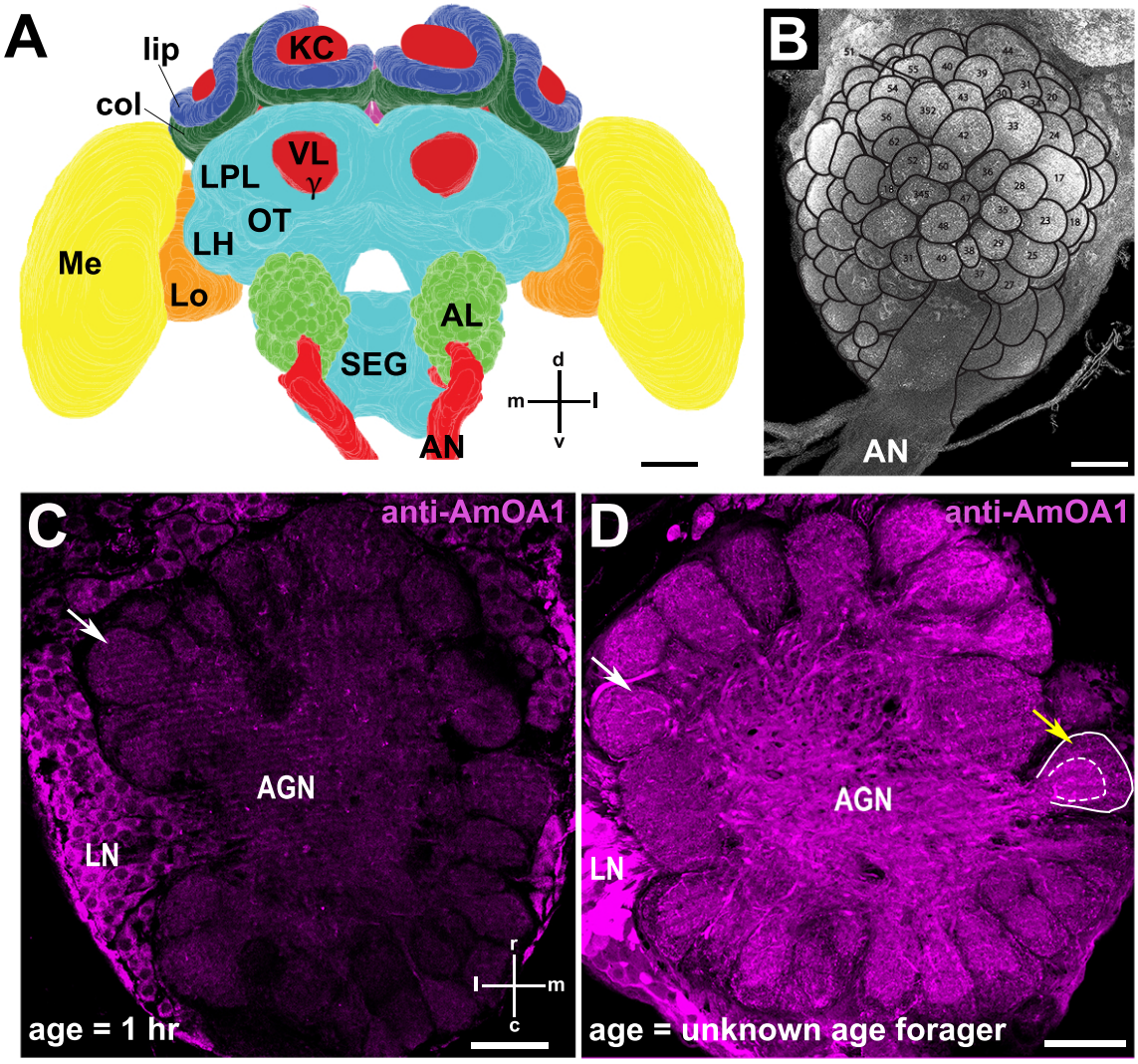


Figure 3.1. Honey bee brain and antennal lobe developmental plasticity in AmOA1. **(A)** Digital reconstruction of the honeybee brain. **(B)** Honey bee antennal lobe immunostained with anti-synapsin with an overlaid digital reconstruction of the antennal lobe with identified dorsal glomeruli (T1-T3). Glomeruli are identified and labeled according to Galizia et al. (1999). Natural variation in anti-AmOA1 staining in a newly emerged adult bee (**C**) and an unknown aged forager (**D**). Arrows denote example glomeruli. Glomerulus outline in D highlights the core and cortex separations of yellow-

arrow labeled glomerulus. Acronyms: KC: mushroom body kenyon cells, lip: mushroom body lip region, col: mushroom body collar region, VL: vertical lobe γ: gamma lobe, LPL: lateral protocerebral lobe, OT: optic tubercle, LH: lateral horn, Me: medulla, Lo: lobula, AL: antennal lobe, SEG: Subesophageal ganglion, AN: Antennal nerve; d: dorsal, l: lateral, v: ventral, m: medial. Scale bar: **A**=250 μm, **B**= 50 μm, **C**= 20 μm, **D**= 20 μm

Newly eclosed adult honey bees show very little expression of the honey bee octopamine 1 receptor (AmOA1) within the glomerular core or cortex (**Figure 3.1C**,—white arrow) or the aglomerular neuropils (**Figure 3.1C**). As a honey bee ages and begins performing foraging behaviors, there is a dramatic change in the AmOA1 receptor expression in which there is a very intense and heterogeneous expression throughout both the glomerular and aglomerular neuropils of the AL (**Figure 3.1D**). For example, the two glomeruli marked by the white and yellow arrows (**Figure 3.1D**) highlight this variation across a single AL. In this unknown aged forager the rostro-lateral glomeruli (white arrow) are generally labeled by anti-AmOA1 in both the core and cortex, however the medio-caudal glomeruli (yellow arrow) instead only have the core labeled by anti-synapsin (**Figure 3.1D**). This variation would likely lead to unique odor and food driven responses within each glomerulus that we hypothesize could be driven by this individual's prior odor-reward experiences.

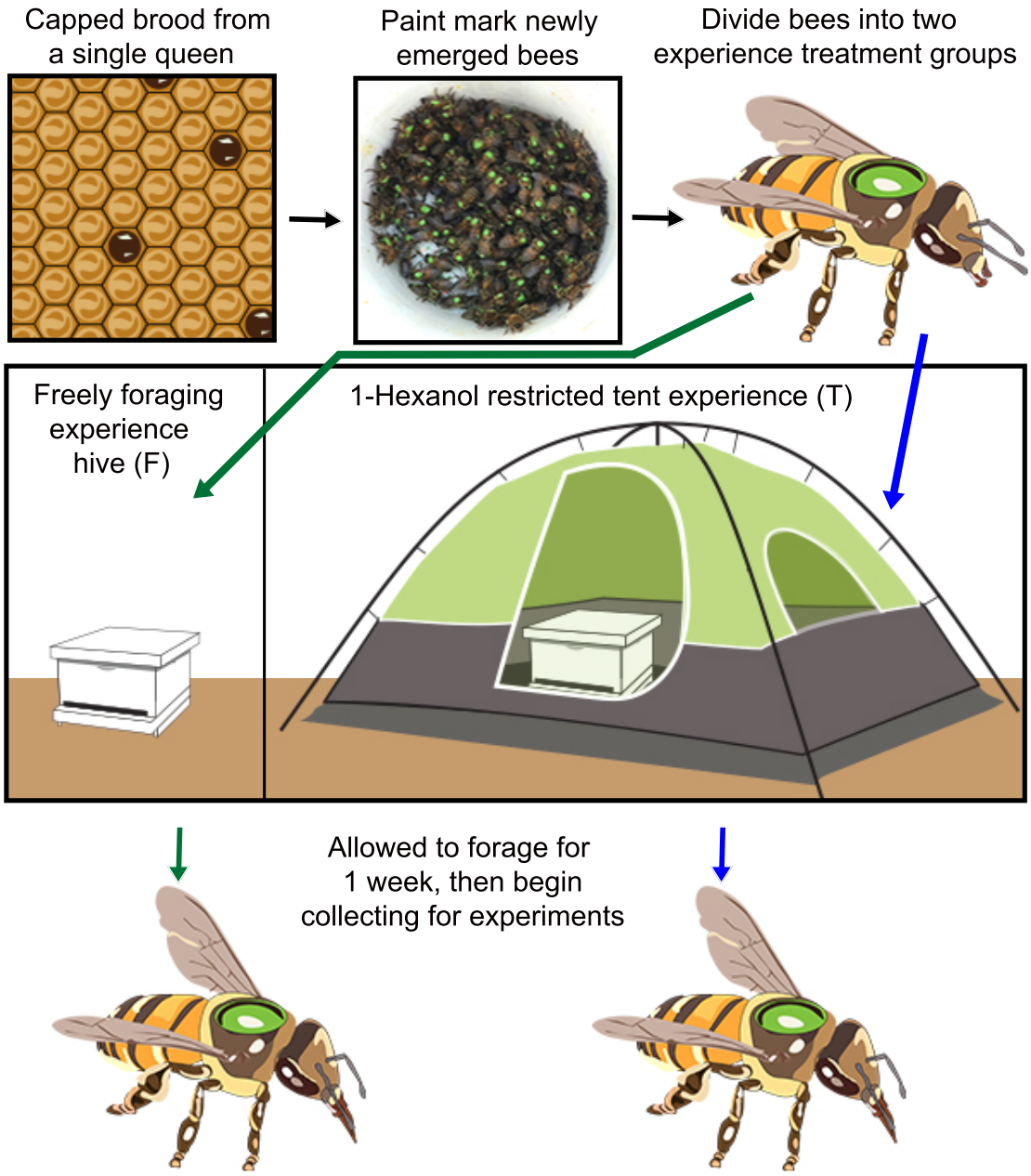
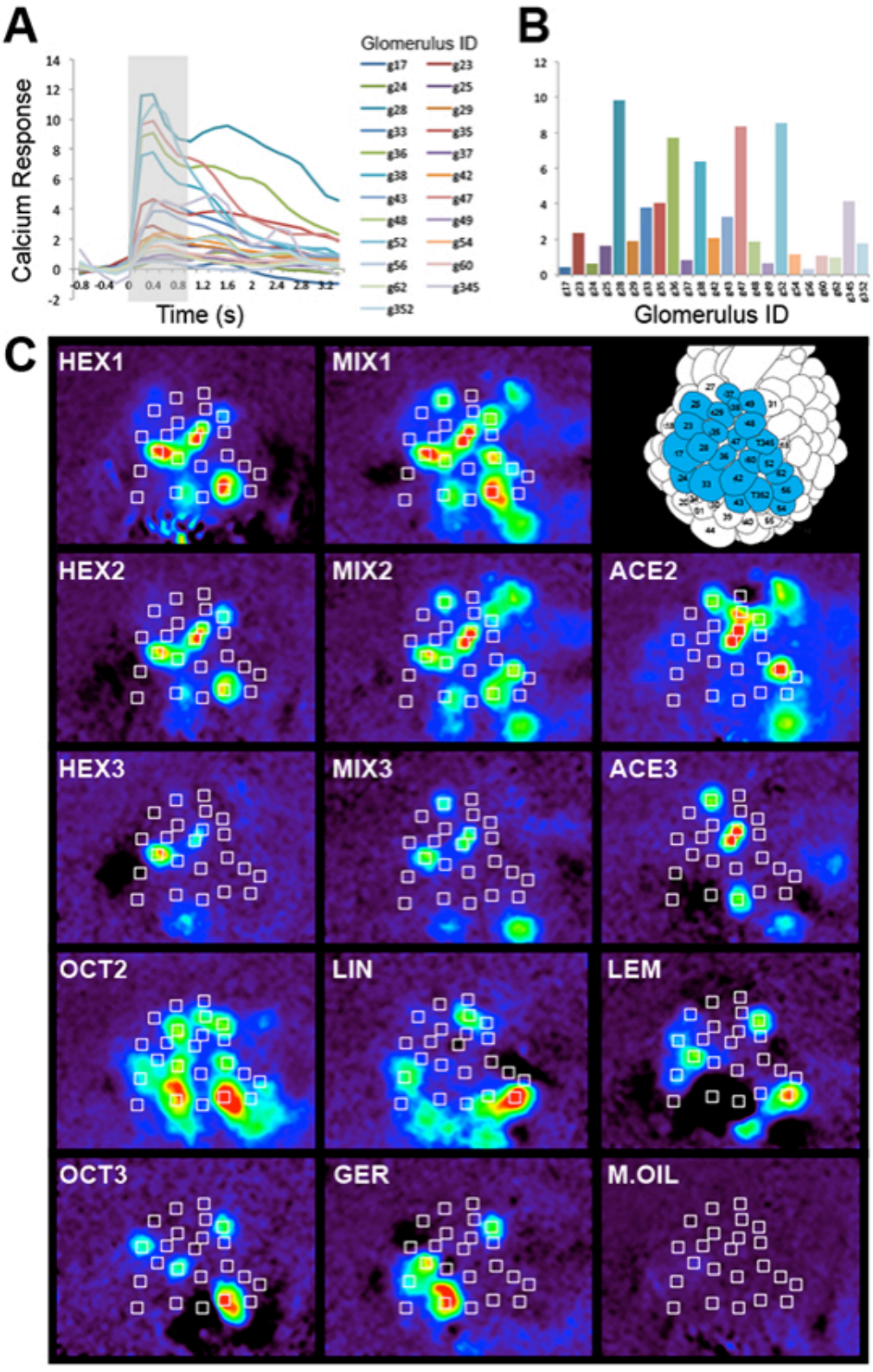


Figure 3.2. Experimental treatments for each experiment. Newly emerged honey bee workers collected from brood frame of a single colony, and paint-marked. Paint-marked workers were split into two treatment hives. The first hive was allowed to forage freely in

the environment (F). The second had all pollen and sucrose resources provided inside of a tent and were associated with 1-Hexanol (T). After foragers had been observed foraging for 1 week, painted foragers were collected for calcium imaging at 31-40 days post eclosion, behavioral experiments (3-11 weeks post eclosion), and immunolabeling (34-40 days post eclosion).

We measured the activity of PNs from the AL using calcium imaging by performing a fura-2 dextran dye injection into a lesion near the lateral antennoprotocerebral (l-APT) tract in the honey bee brain for bees with a freely foraging (F) or those with a tent restricted (T) experience (Locatelli et al., 2013; Sachse & Galizia, 2002). We visualized the glomerular responses of the rostro-dorsal portion of the honey bee antennal lobe (**Figure 3.3**). Each recording trial lasted 10 seconds. Odor stimulation lasted 1 second and began 3 seconds after the onset of recording. We presented 13 olfactory stimuli and the mineral oil solvent to each bee while recording the activity of 23 glomeruli (**Figure 3.3**), for 10 freely foraging (F, n=10) and eight tent-restricted bees.


A
Calcium Response
Time (s)
Glomerulus ID
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g42
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g47
g48
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g62
g345
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B
Glomerulus ID
C
HEX1
MIX1
HEX2
MIX2
ACE2
HEX3
MIX3
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OCT2
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LEM
OCT3
GER
M.OIL

Figure 3.3. Calcium imaging data collection and processing protocol, example data from a single freely foraging individual. (**A**) Response of 23 identified glomeruli (colored lines) to Mix1 over time. Shaded region denotes odor stimulation. (**B**) Averaged response during odor stimulation (shaded region) for each glomerulus in A. (**C**) Example false colored maximal response of the honey bee antennal lobe to each odor stimulus presented. Schematic reconstruction of the rostral portion of the antennal lobe and the 23 identified glomeruli imaged in this experiment in top right panel. Odors labels: HEX1 = $2x10^{-2}$ M 1-Hexanol, HEX2= $1x10^{-2}$ M 1-Hexanol, HEX3= $1x10^{-3}$ M 1-Hexanol, MIX1= $2x10^{-2}$ M 1-Hexanol and $1x10^{-2}$ M Acetophenone, MIX2= $1x10^{-2}$ M 1-Hexanol and $1x10^{-2}$ M Acetophenone, MIX3= $1x10^{-3}$ M 1-Hexanol and $1x10^{-3}$ M Acetophenone, ACE2= $1x10^{-2}$ M Acetophenone, ACE3= $1x10^{-3}$ M Acetophenone, OCT2= $1x10^{-2}$ M 2-Octanone, Oct3= $1x10^{-3}$ M 2-Octanone, GER= $1x10^{-2}$ M Geraniol, LEM= $1x10^{-2}$ M Lemon oil, LIN= $1x10^{-2}$ M Linalool. All odors dissolved in mineral oil (M.OIL).

Each odor was presented two times per bee. The glomerular responses were highly consistent between presentations within an animal (Response profile correlations: F= 0.879 +/- 0.028; T= 0.896 +/- 0.0119). However, the precise glomerular response profiles to a panel of odors were variable across animals (**Figure 3.4 A,B**). We observed some consistency across freely foraging animals that may have some similar olfactory experiences if they had been foraging at the same food sources. For example, on average, glomerulus 36 responded to 2-octanone (**Figure 3. 4A**) and glomerulus 37 responded to acetophenone (**Figure 3.4B**). However, the odorants to which glomerulus 36 and 37

responds were variable between animals. Glomerulus 36 in animal 1 responded to both 1-hexanol and acetophenone in addition to 2-octanone, and in animal 2, glomerulus 36 also responded to linalool and lemon oil (**Figure 3.4A**). Additionally, glomerulus 37 in animal 2 also responded to 1-hexanol, 2-octanone and linalool (**Figure 3.4B**).

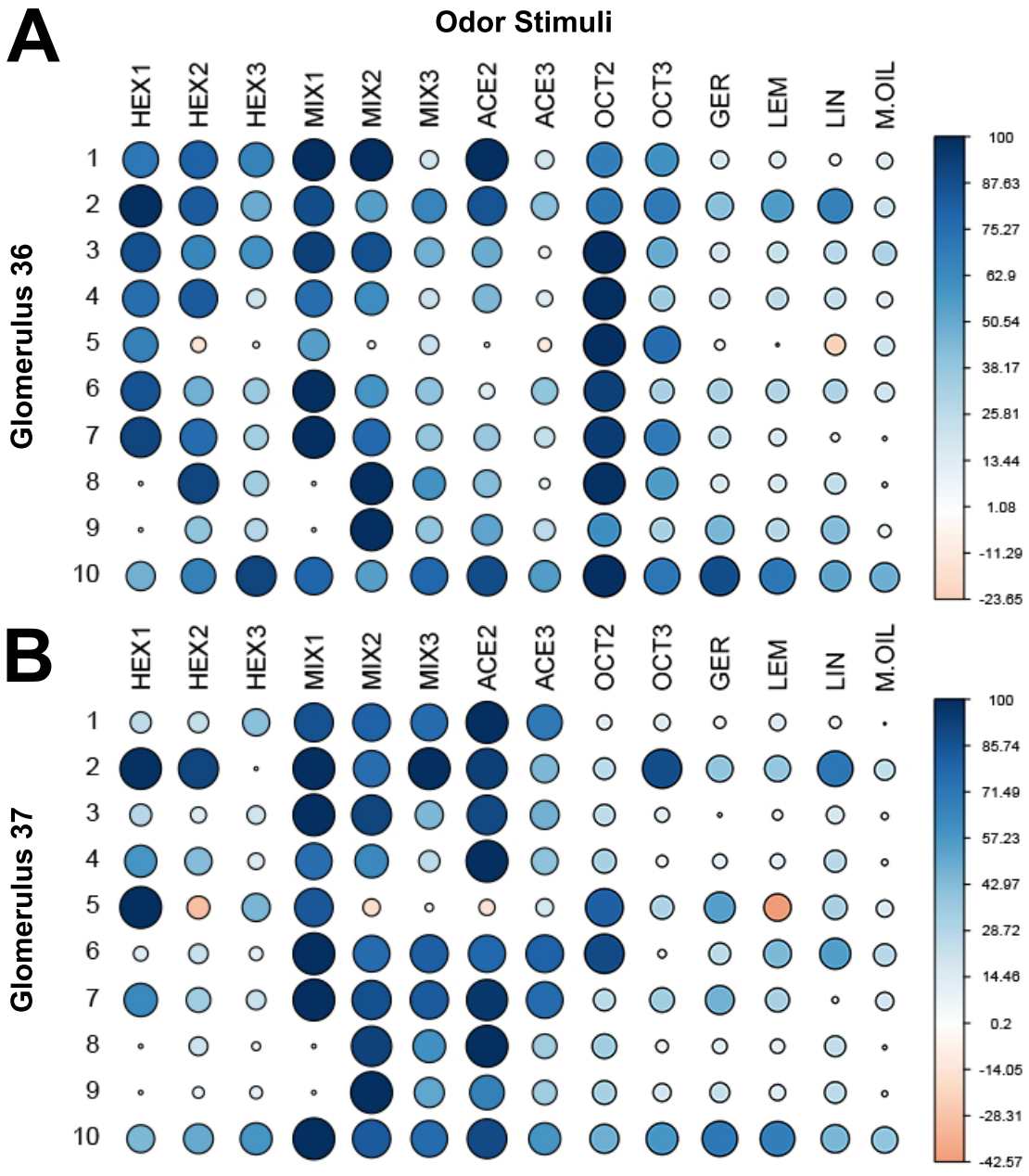


Figure 3.4. Natural inter-individual variation in glomerular response profiles to odors in freely foraging honey bees. Response profiles for glomerulus 36 (**A**) and glomerulus 37 (**B**) for 10 age-matched foragers with unknown olfactory experience. Size of circle and color denotes the magnitude of calcium response and direction of change respectively. Odors labels: HEX1 = $2x10^{-2}$ M 1-Hexanol, HEX2= $1x10^{-2}$ M 1-Hexanol, HEX3= $1x10^{-3}$ M 1-Hexanol, MIX1= $2x10^{-2}$ M 1-Hexanol and $1x10^{-2}$ M Acetophenone, MIX2= $1x10^{-2}$ M 1-Hexanol and $1x10^{-2}$ M Acetophenone, MIX3= $1x10^{-3}$ M 1-Hexanol and $1x10^{-3}$ M Acetophenone, ACE2= $1x10^{-2}$ M Acetophenone, ACE3= $1x10^{-3}$ M Acetophenone, OCT2= $1x10^{-2}$ M 2-Octanone, Oct3= $1x10^{-3}$ M 2-Octanone, GER= $1x10^{-2}$ M Geraniol, LEM= $1x10^{-2}$ M Lemon oil, LIN= $1x10^{-2}$ M Linalool. All odors dissolved in mineral oil (M.OIL).

In order to measure the degree of inter-individual variation we used a jackknife resampling method and created all possible non-repeating 7x7 correlation matrices, where each cell in each correlation matrix was the correlation of the mean glomerular response value (either for an individual glomerulus or a combined vector of all glomeruli) to all odors between two individual bees within the same treatment group. We then took the determinant value of each of these correlation matrices, which reflected the degree of correlation or variability within each matrix (**Figure 3.5A,B**). The determinant score value is an inverse of the overall correlation value, close to zero being more correlated (less variable) and close to 1 being more uncorrelated (more variable) (**Figure 3.5A,B**). This determinant score measure was also sensitive to the size of the correlation matrix

used, therefore we needed to compare matrices of the same size i.e. 7x7 matrices for both the tent restricted (T) and the freely foraging (F) bees. Thus we ended up with 8 (T) and 120 (F) determinant score values in each of our comparisons. Using this measure we first compared the response profiles of the individuals in each treatment group while including the responses of all glomeruli to all odors in a single vector. We found that there was significantly lower inter-individual variation in the tent restricted (T) bees than the freely foraging (F) bees (Mann-Whitney U test, $W= 857$, $p<0.001$, T Det. mean = 0.0276, F Det. mean = 0.0524).

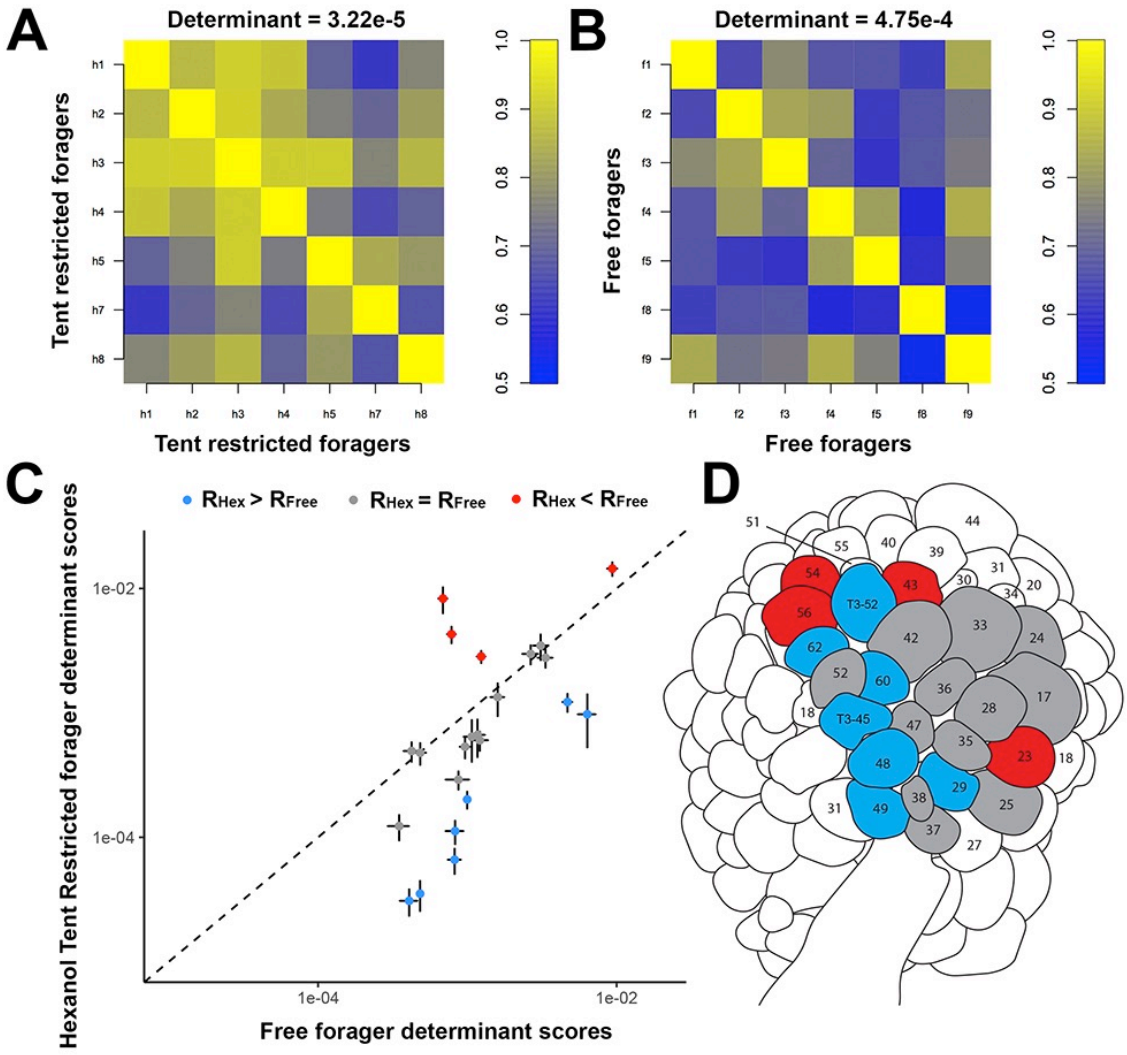


Figure 3.5. Reduced inter-individual variation in glomerular response profiles to odors. Example correlation matrices and determinant scores for how glomerulus T3-52 responses to all measured odors, in which each square represents the correlation of the responses of T3-52 to all odors between to different individual bees, in one sample of (**A.**) 7 bees with a 1-hexanol tent- restricted experience, and (**B.**) 7 bees with a freely foraging experience. **C**. Regression of the distributions of response profile determinant scores for each glomerulus. Each point is the determinant score distribution for an

identified glomerulus for the free foraging experienced bees plotted against the distribution for the same glomerulus in the 1-hexanol tent restricted experience bees. The dashed line denotes a slope of 1 and intercept at the origin. Colors denote significant differences between experience treatments. **D**. Reconstruction of the honey bee antennal lobe and highlighted glomeruli denoting significant differences between experience treatments. Statistics: false-discovery rate corrected Mann-Whitney U tests, $p<0.05$, Hex<Free (blue), Hex>Free (red).

We next wanted to determine if the reduced inter-individual variation was driven by all of the 23 measured glomeruli or, alternatively, by only a few glomeruli that change significantly between individuals. Therefore, we compared the determinant scores as before, however now only including the values for a single glomerulus in each correlation matrix (8 T and 120 F) and repeating this comparison for each glomerulus and each treatment group (Ex. **Figure 3.5A,B**). As expected by our initial analysis, the regression of the determinant scores for the freely foraging bees versus the tent restricted foragers tends to fall below the 1:1 (F : T) line (**Figure 3.5C**). However, the observed reduction in overall variation is driven by a significant reduction in just 7 of the measured 23 glomeruli (**Figure 3.5C**, false-discovery rate corrected Mann-Whitney U tests, $p<0.05$, Tent<Free highlighted blue). Additionally, we found 4 glomeruli that show the opposite pattern, higher inter-individual variation in the tent-restricted compared to the free foragers (**Figure 3.5C**, false-discovery rate corrected Mann-Whitney U tests, $p<0.05$, Tent>Free highlighted red). Thirteen of the 23 glomeruli show no significant difference

in the inter-individual variation between the two experiential treatments (**Figure 3.5C**, false-discovery rate corrected Mann-Whitney U tests, $p>0.05$, Tent = Free highlighted grey). However, some of this last group also includes glomeruli that are typically inactive during most of the odor stimuli we presented, such as glomerulus 17 (**Figure 3.5C**).

Experience-dependent effects on odor mixture learning

We next used a computationally taxing behavioral assay to compared the two odor experience groups (T and F) (Wright & Smith, 2004), in hopes of revealing potential behavioral effects due to experience. The T and F groups were split into two subgroups (Mix1: PAA, Non, Far; or Mix2: Ace, Ger, 2-Oct). Each subgroup was further divided into receiving either a *variable* mixture protocol (**Figure 3.6A**) or a *constant* mixture protocol (**Figure 3.6B**). Both groups then received 16 presentations of the odor mixture—either a variable (**Figure 3.6C**) or a constant protocol (**Figure 3.6D**)—paired with sucrose. We then tested each bee for a proboscis extension response to the individual components that made up their associated mixture 2 hours later (**Figure 3.6A-D**; See methods and Wright & Smith, 2004 for further details).

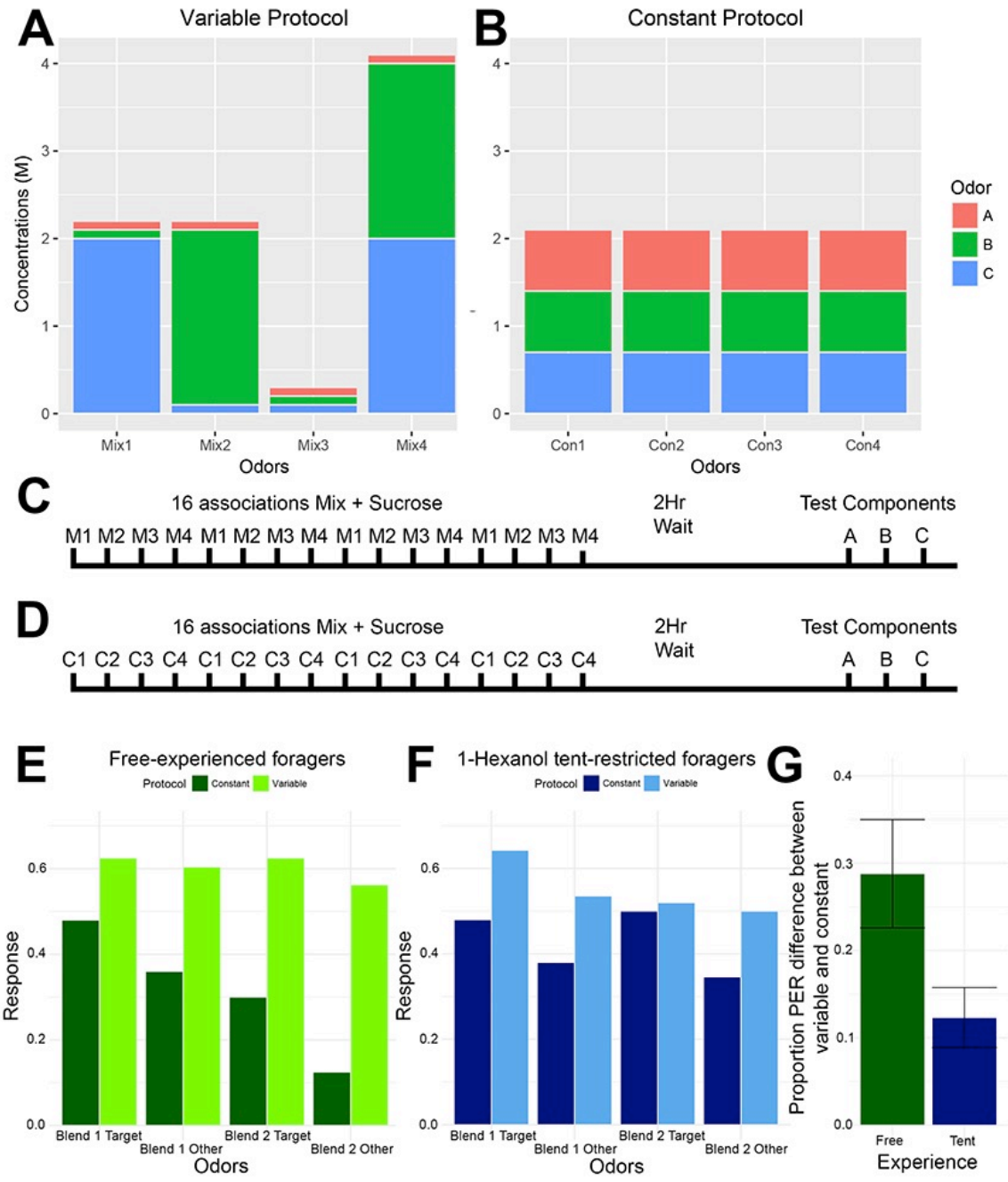


Figure 3.6. Odor mixture variance assay and the 2 hr memory test results of honeybee workers aged 4-11 weeks post adult emergence. The odor mixture component concentrations used during the (**A**) variable mixture protocol and (**B**) the constant mixture protocol. (**C**) A schematic of the associative conditioning and component memory testing

for variable mixture protocol. Bees received different mixtures (Mix1, Mix2, Mix3 or Mix4) in a pseudorandomized order over 16 associative acquisition trials. (**D**) A schematic of the associative conditioning and component memory testing for constant mixture protocol. Bees received identical mixtures (Con1= Con2= Con3= Con4) over 16 associative acquisition trials. Memory test component order was randomized between bees for both protocols. We use two sets of mixture components: mixture blend 1 (A= Phenylacetaldehyde, B= Nonanal, C= α-farnesene) and mixture blend 2 (A= Acetophenone, B= Geraniol, C= 2-Octanone) in which A is the target odor that is held at the constant intensity in the variable protocol. See methods and Wright and Smith (2004) for more detail. (**E-F**) Bees were trained using either a constant mixture protocol (dark color) or a variable mixture component protocol (light color). The proportion of bees extending their proboscis 2 hrs after conditioning to a mixture component target (A, intensity held constant for the variable mixture odors throughout all acquisition trials) and the other components (B and C, intensity varied throughout acquisition trials) for both mixture blends. (**E**) Bees with a natural-foraging experience (green). (**F**) Bees with a 1-Hexanol tent-restricted foraging experience (blue). (**G**) Difference between the responses of bees trained using the variable mixture protocol and the constant mixture protocol for bees with a free foraging experience (green) and a 1-Hexanol tent restricted foraging experience (blue). Error bars represent standard error from the mean. Statistics: (**E-F**) GLMM with interacting factors of mixture odors (*Brassica* or Replicate), Odor component (PAA, Non, α-Far, Ace, Ger, 2-Oct), Bee experience (free vs. restricted),

training protocol (Constant vs. variable), and Bee identity as a random factor. (**G**) Mann-Whitney U test, W=1, p <0.01.

Bees with a tent-restricted experience were able to associate mixture stimuli with sucrose just as well as freely foraging adults over the 16 acquisition trials (**Figure 3.S1**, GLM, family= Binomial, Experience*Mixture: Z=1.389, p=0.165, Experience*Protocol: Z= -1.339, p=0.181, Experience*Mixture*Protocol: Z=-1.762, p=0.078). As might be expected, it took bees in the variable odor mixture associations more trials to begin responding than bees that received the constant protocol (**Figure 3.S1**, GLM, family=Binomial, Variable protocol, Z= -4.702, p<0.001). Bees with the tent-restricted experience associated odors more quickly (**Figure 3.S1**, GLM, family=Binomial, Experience, Z= 5.678, p<0.001).

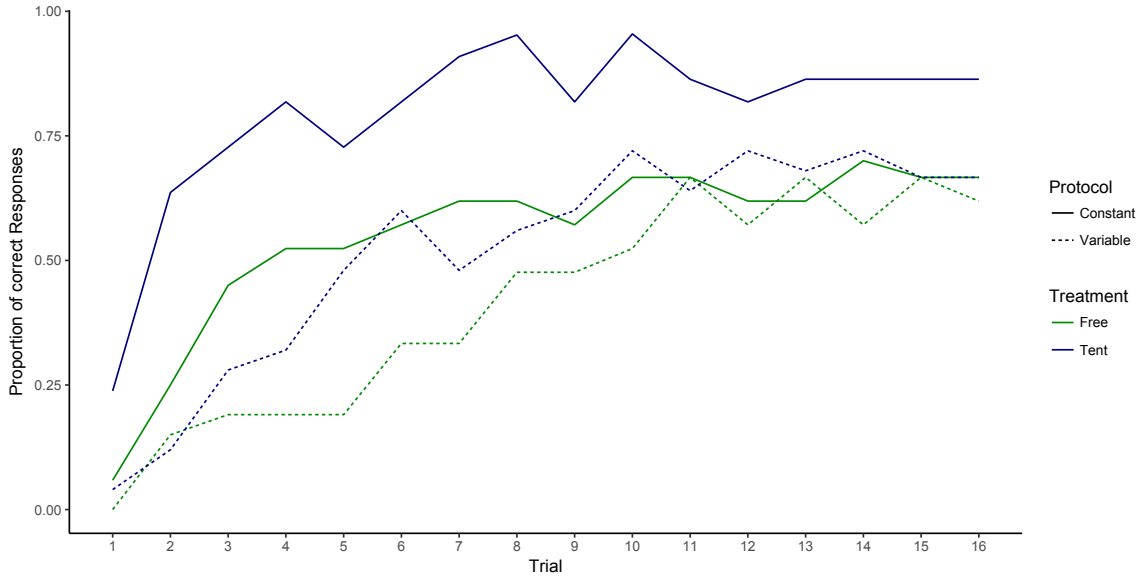


Figure 3.S1. Acquisition of odor mixture blend 1 across freely foraging (green) and tent bees (blue) which received either a constant (solid line) or a variable (dashed line) mixture protocol. Free Variable N=21, Free Constant N=21, Tent Variable N= 25, Tent Constant N=22.

We next tested the components of each odor mixture, 2 hours later in a memory test. The tent-restricted bees generalized across all mixture components during the 2-hour memory test, even when trained to the constant mixture protocol (**Figure 3.6F**, GLMM, family=Binomial, BeeID random factor, Experience*Protocol, $t=-2.819$, $p<0.01$). The freely foraging bees only generalized across mixture components when trained to a variable protocol (**Figure 3.6E**). We compared the difference between the variable and constant mixture protocols for each odor and found that the difference was significantly smaller for the tent bees than the freely foraging bees (**Figure 3.6G**, Mann-Whitney U test, $W=1$, $p<0.01$). Thus, the bees with reduced olfactory experience were somehow processing odor mixtures differently than their freely experienced counterparts. We otherwise found similar responses to that reported in Wright and Smith (2004) for both Mixture blend 1 and Mixture blend 2. The target odor—which was held constant in the variable mixture— elicited a higher response than the variable components (**Figure 3.6E**, GLMM, family=Binomial, BeeID random factor, Ace: $t=2.544$, $df=457$, $p=0.01$, PAA: $t=2.256$, $df=457$, $p=0.025$; other odors $p>0.05$). Additionally, in general there was a higher response to each of the mixture components when bees were trained to a variable

mixture than to a constant mixture (**Figure 3.6E**, GLMM, family=Binomial, BeeID random factor, Protocol, t=4.385, df=458, p<0.001).

Experience-dependent effects on the antennal lobe network

In order to explore the network changes that occur as a result of our experiential treatments, we immunolabeled the brains of foragers in our two treatment groups as well as newly emerged adult bees from the same cohort used to establish the colonies. Immunolabeling of antibodies was against the honey bee octopamine1 receptor (anti-AmOA1) and the structural protein polymerized F-actin using Rhodamine Phalloidin.

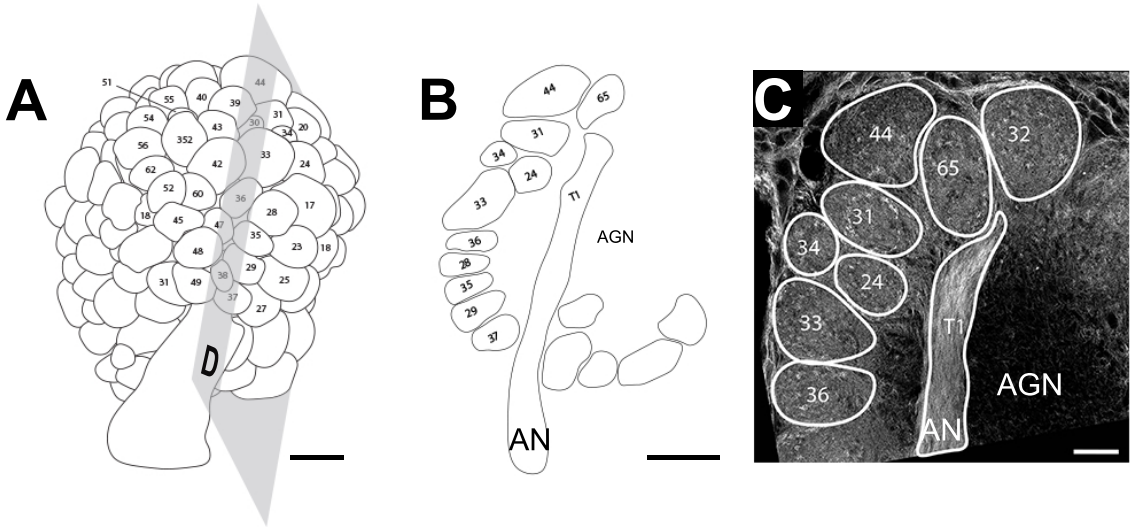
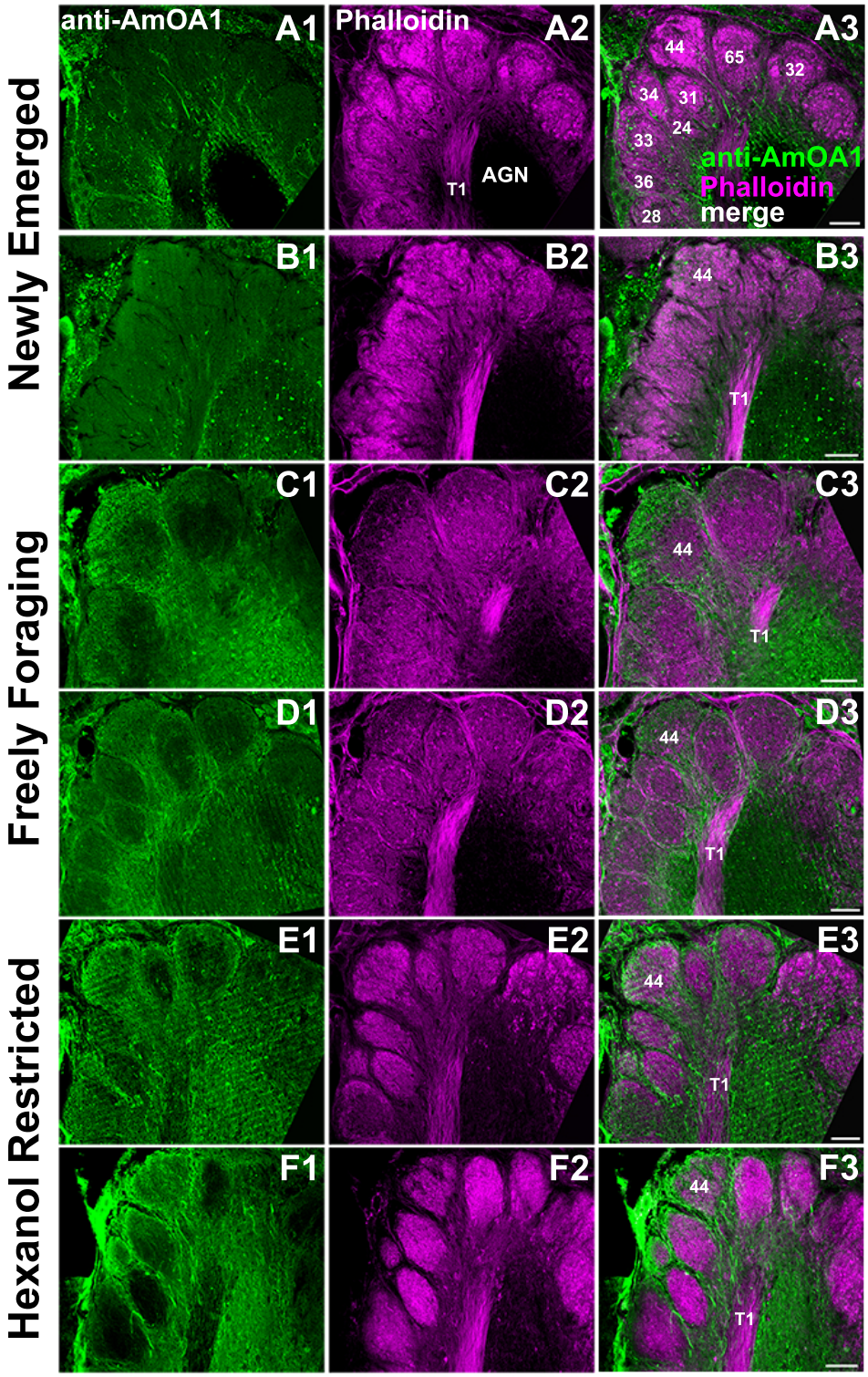


Figure 3.7. (A) A digital reconstruction of the antennal lobe in (Figure 1B) Glomeruli are identified and labeled according to Galizia et al. (1999). Shaded plane illustrates sagittal section displayed in B. **(B)** A digital, sagittal section taken from the reconstruction in A. Section clearly identified by the T1 antennal nerve track. **(C)** A digital overlay with labeled glomeruli and T1 antennal nerve track on sagittal sectioned antennal lobe. Tissue has polymerized actin immunostained with Rhodamine Phalloidin. AN: Antennal nerve,

AGN: aglomerular neuropil of the antennal lobe. Scale bar: **A**= 50 μm, **B**= 50 μm, **C**= 25 μm.

We compared sagittal sections of the honey bee antennal lobe in which the T1 tract of the antennal nerve was visible and distinct glomeruli, such as 44, were present to ensure we were comparing similar synaptic regions (**Figure 3.7A-C**). As previously observed (**Figure 3.1C,D**), there is a large shift in the expression patterns of AmOA1 across antennal lobe development from newly emerged adult (**Figure 3.8A1,B1**) to aged forager (**Figure 3.8C1,D1**). We observed very little anti-AmOA1 staining (green) in newly emerged adult bees (**Figure 3.8A1,B1**). Any staining present was at very low levels and was homogeneously distributed across the aglomerular and glomerular regions. In the aged freely foraging adults, there was much more anti-AmOA1 staining, which was characteristically heterogeneous across the aglomerular and glomerular neuropils as well as between glomeruli within the tissue and between individuals (**Figure 3.8C1,D1**; Sinakevitch et al. 2011). For example, some individuals show uniform staining in glomerulus 44 in C1 and D1, however this uniformity was not consistent across all individuals in the freely foraging treatment (**Figure 3.8**).


Newly Emerged
Freely Foraging
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anti-AmOA1
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Phalloidin
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28
anti-AmOA1
Phalloidin
merge
T1
AGN
B1
B2
B3
C1
C2
C3
D1
D2
D3
E1
E2
E3
F1
F2
F3

Figure 3.8. Sagittal sections of honeybee left antennal lobe with different degrees of olfactory experience. Left most panel column immunostained with anti-AmOA1 (1), middle panel column immunostained polymerized actin using Rhodamine Phalloidin (2), right-most panel column merged anit-AmOA1 and Rhodamine Phalloidin immunostaining. **(A-B)** Newly emerged adult honeybee workers. **(C-D)** Adult forager honeybee workers aged 4-5 weeks post adult emergence. **(E-F)** Adult forager honey bees age-matched and sisters to C & D enclosed in a tent with 1-Hexanol associated with all food resources (sucrose and pollen) for their entire adult life. Glomeruli are numbered in A3 and glomerulus 44 and T1 antennal nerve track are labeled in all overlays for reference. Scale bars: 25 μm.

Within the tent treatment (T), we also observed a heterogeneous staining pattern against AmOA1 across the aglomerular and glomerular neuropils, similar to that observed in bees with natural foraging experience. Overall, there were no obvious or consistent anti-AmOA1 staining patterns we could observe across or within the two aged forager groups, irrespective of odor experience. This finding is additionally supported by the lack of difference in the acquisition phase of odor learning (**Figure 3.S1**), as AmOA1 is necessary for odor-reward associative learning (Farooqui et al., 2003; Farooqui et al., 2004).

We also stained all animals using Rhodamine Phalloidin (magenta), which binds to polymerized F-actin (**Figure 3.8**). Polymerized F-actin is highly correlated with dendritic

plasticity and is found in high concentrations in axonal growth cones, and during spine outgrowth including in the honey bee brain (Frambach, Rössler, Winkler, & Schürmann, 2004; Groh, 2005; Groh et al., 2012; Okamoto, Nagai, Miyawaki, & Hayashi, 2004; Rössler, Kuduz, Schürmann, & Schild, 2002; S. J. Smith, 1988). We observed a very intense staining of polymerized F-actin within the glomeruli of the newly emerged adult bees (**Figure 3.8A2,B2**). This staining pattern is absent in all preparations of the glomeruli of the freely foraging, aged bees (**Figure 3.8C2, D2**). Freely foraging bees have a much more porous and sparse polymerized F-actin staining within their glomeruli (**Figure 3.8C2, D2**). Surprisingly, the tent bees have very homogeneous and intense polymerized F-actin staining across all glomeruli (**Figure 3.8E2, F2**). Unlike their naturally foraging, age-matched sisters (**Figure 3.8C2, D2**), the tent-restricted bees' polymerized F-actin staining patterns are strikingly similar to that observed in the inexperienced, newly emerged adult bees (**Figure 3.8A2, B2**).

Given this information we further hypothesized that the antennal lobes' local neurons could be over-synapsing, or over-connecting, all glomeruli and thus homogenizing the responses to all odors and reducing the inter-individual variability in the glomerular response profiles to odors, as well as affecting odor discrimination in the odor mixture variance recall assay. To test this we compared the glomerular tuning profiles (**Figure 3.3B**), or sharpness of the glomerular pattern on the antennal lobe, for individuals within each treatment group. If there had been over-synapsing in bees with reduced experience, we would expect to see a more homogeneous glomerular response to odor stimuli and,

thus, the slope of their glomerular tuning profiles—the glomerular calcium response across glomeruli for each odor—should be much closer to zero, compared with their experienced sisters. However, when we made this comparison, we observed no difference in the glomerular tuning profile slopes between the two experiential groups (ANCOVA, $p>0.05$). Thus, there are likely much more subtle network changes occurring as a result of experience reduction, which cannot be explained by changes in the synaptic connectivity of a single neuronal sub-population.

DISCUSSION

Effect of olfactory restriction

Bees with natural foraging experiences may have foraged at multiple floral species, each of which has complex olfactory stimuli—up to ~100 different volatile chemicals are present at floral nectaries (Levin, McDade, & Raguso, 2003; Raguso, 2008). In our odor-restriction treatment, bees only have access to a single odorant consistently associated with the resources they collected. We have shown that by reducing the complexity of stimuli and controlling the olfactory experience of bees, there is an overall reduction in the inter-individual variation in how antennal lobe projection neurons respond to odors (**Figure 3.5C**). The chronic activation—or lack thereof—of certain glomeruli during sucrose reinforcement could have global effects on the synaptic weights of interneuron connections (LN-LN and LN-PN) in the antennal lobe, which could explain this result. This is supported by the high levels of polymerized actin staining in tent bees, indicative of active structural or synaptic plasticity similar to that observed in newly eclosed bees

(**Figure 3.8-**Rhodamine Phalloidin) (Frambach et al., 2004; Groh, Ahrens, & Rössler, 2006). Furthermore, bees with a restricted experience respond similarly during recall to all the components of an associated mixture, even when receiving an association protocol that should produce strong responses to only one of the components (**Figure 3.6G**). This is further supported by Cabirol et al. (2017) which recently found that experience deprivation results in a reduced ability to perform more complex learning tasks such as reversal learning.

Pupal olfactory input on antennal lobe formation

There is a critical period in antennal lobe development pre-eclosion, in which olfactory sensory neuron input is necessary for both glomeruli formation in insects (Hildebrand, Rössler, & Tolbert, 1997). In brief, axonal projections from olfactory receptor neurons to the antennal lobe initiate the formation of preglomeruli (protoglomeruli in *Manduca*) (Groh, 2005; Hildebrand et al., 1979; Hildebrand et al., 1997; Oland et al., 1990). When animals are deprived of olfactory receptor neuron inputs during pupal development, the antennal lobes develop without a defined glomerular structure (Hildebrand et al., 1979; Oland & Tolbert, 1987). After preglomeruli formation, glial cells form a boundary that will later become the full glomeruli (Oland et al., 1990; Oland & Tolbert, 1987, 1989). At this stage, but before the full synaptic glomerulus is formed, some projection neurons begin to arborize into their glomerular targets (Oland et al., 1990). The adult glomeruli can be visualized with anti-synapsin staining as the mature synapses form between the olfactory receptor neurons, local neurons, and projection neurons (Oland et al., 1990;

Oland & Tolbert, 1989). More recently, it has been shown via genetic knockout of the co-receptor to all olfactory receptors in the olfactory receptor neurons of two ant species—*Harpegnathos saltator* and *Ooceraea biroi*—that normal development of the antennal lobe requires not only olfactory receptor neuron axon presence, but also olfactory responses of those olfactory receptor neurons (Trible et al., 2017; Yan et al., 2017).

Post-eclosion input on antennal lobe maturation

Post-eclosion experience shapes the development of neural networks in bees in the visual processing centers of the mushroom bodies (Coss et al., 1980; Fahrbach et al., 1995; Farris, 2005; Farris et al., 2001) and to some degree the early network development of the antennal lobe (Brown et al., 2004; Brown et al., 2002). Hourcade et al. (2009), Fernandez et al. (2009), and Locatelli et al. (2013) showed there are physiological changes in how the antennal lobe responds to odors associated with short-term olfactory experience, by which the projection neuron responses to odors and odor mixtures are modified to allow for a more clear discrimination of relevant odors in the antennal lobe. Prior to the present work, in experiments with an experience-associated treatment, experimenters only isolated behavioral honey bee castes which did or did not have in-hive experience (Brown et al., 2004; Brown et al., 2002; Coss et al., 1980; Fahrbach et al., 1995; Farris, 2005; Farris et al., 2001; Hourcade, Muenz, Sandoz, Rössler, & Devaud, 2010; Hourcade, Perisse, Devaud, & Sandoz, 2009). They then measured physiological changes or region-specific morphological changes in volume and synaptic densities (Brown et al., 2004; Brown et al., 2002; Coss et al., 1980; Fahrbach et al., 1995; Farris, 2005; Farris et al.,

2001). In the present study, we add a more comprehensive view of the effects of olfactory restriction at 3 levels: physiological responses to odors, odor mixture recall, and the maturity of the antennal lobe network using immunostaining.

Our data provide a more comprehensive picture of the post-eclosion, experience-dependent effects of odor processing in bees. In light of previous work, our data suggest that experience post-eclosion is also necessary for the production and/or maintenance of a mature antennal lobe network that is capable of performing complex olfactory-based tasks. We do not yet know the identity of the specific neuronal sub-types and network connections affected during olfactory restriction, which warrants further study. However, it is likely affecting more than one sub-population of neuron within the antennal lobe and could possibly also affect centrifugal (neuromodulatory) inputs from higher-order brain regions.

In our treatments we manipulated the chronic food-odor associations that adult honey bee foragers had access to. The tent-restricted bees had access to all of the normal colony odors, thus, the primary olfactory difference between groups was the diversity of food-associated odors that they experienced. However, we introduced additional differences when we tent-restricted bees. In addition to olfactory experience, we manipulated the general foraging experience of bees when we restricted flight distance and aerobic effort, visual diversity, nectar complexity, and likely the colony forager dance communication profile. Most of these effects likely have significant impacts on other brain regions,

specifically the visual system. However, we cannot rule out differences due to centrifugal inputs to the antennal lobe from other brain regions, a possibility that warrants further study. We did not directly measure hive temperatures between the two treatments, however honey bees are incredibly efficient thermoregulators and are capable of maintaining optimal homeostatic temperatures in the hive between a large range of temperatures with sufficient access to water and sugar resources, which both groups of bees had ready access to (Cook & Breed, 2013; Egley & Breed, 2013; Fahrenholz, Lamprecht, & Schricker, 1989; Jones & Oldroyd, 2006; Kühnholz & Seeley, 1997; Siegel, Hui, Johnson, & Starks, 2005; Starks & Gilley, 1999).

Perception of complex odor mixtures

The differences in recall of odor mixtures by bees with a restricted vs unrestricted experience could have several possible explanations that are worthy of further study. First, bees with a restricted experience could have difficulty recognizing odors in general. These bees could also have less precise recall of odor mixtures compared to bees with unrestricted experience, or they could simply have a higher degree of generalization to odor associations. These possibilities could be tested using a discrimination task across a number of odorants followed by a recall test to similar and dissimilar odorants as done in Guerrieri et al. (2005). The coding of complex olfactory mixtures is not well studied in the antennal lobe, despite these stimuli being abundant in natural settings (Guerrieri, Lachnit, Gerber, & Giurfa, 2005; Laloi et al., 2000; Locatelli et al., 2016; Strutz et al., 2014). More complex olfactory stimuli would likely be computationally more intensive to

process; therefore, it is likely that if the antennal lobe were somehow underdeveloped (as our data suggest), processing mixtures could be more difficult for odor-restricted bees compared to their naturally foraging counterparts.

Summary

Our data show that post-eclosion olfactory experience affects the neuroanatomical structure of the antennal lobe network, which has an impact on physiological processing of odors and odor-mixture recall. This work highlights the importance of individual experience on olfactory processing. To fully elucidate the functions of the antennal lobe and olfactory bulb, more research is needed that not only focuses on individuals with a homogeneous experience, but also includes individuals with a diversity of experiences.

METHODS

Bee keeping and rearing

A single honey bee queen (*Apis mellifera carnica*) was caged on an empty frame for 2 days and allowed to lay eggs on a single frame. After egg laying, the queen was released to move freely in the hive. Just prior to adult emergence, the frame was placed in an incubator. Newly emerged (eclosed) adult honey bees were collected from the frame within 24 hours of eclosion and were paint marked with a non-toxic pen (Sharpie, oil based). A total of 1000 newly emerged bees were marked and split into 2 groups of approximately 500 bees every 2-4 weeks. Each of these groups of ~500-700 of bees was introduced into one of two 10-frame queenright host hives, named by T and F. We used

one pair of host hives for both the calcium imaging and immunohistochemical experiments, and two pairs of host hives for the behavioral experiments.

The hives were placed within 30 meters of one another in a shaded courtyard. Hive T was enclosed by an individual 15.9 m^3 (8x10x7ft) tent in which all of the sucrose and pollen resources were artificially provided. The second hive (F) was left outside of the tents and foraged freely at the Tempe, AZ campus of Arizona State University. (**Figure 3.2**).

The food resources in the tents were marked with a single-odor stimulus 1-hexanol (hive T) as an artificial CS. This was done as follows: The 50% sucrose (w/w) solutions provided to the tent bees contained 0.01% (w/w) odor. Around both the sucrose and pollen resources, a 10% odor solution diluted in mineral oil was applied to an absorbent material placed around both resources. Sucrose solutions were replaced every 24 hours and a new 10% odor mixture was placed around the resources once in the morning and once in the afternoon. To the human nose, all materials retained the smell of the odor, even after 24 hours.

Honey bee collection

Paint-marked honey bee foragers were all collected when returning to the hive from a foraging event approximately 7 days after the first paint-marked bee was observed foraging.

Immunostaining collection

Paint-marked bees from all hives were collected simultaneously over three days and immediately processed for immunostaining. These bees were between 34-40 days old from adult emergence.

Calcium imaging collection

Paint-marked bees were collected from all hives simultaneously over a three-week time frame and were immediately harnessed and prepared for calcium imaging. The honey bee cohort used in experiments were switched from the original 1000 newly emerged bees to a cohort 2-4 weeks younger half way through the experiment, in order to keep the length of foraging experience more consistent across bee subjects. All bees were between 31-40 days old from adult emergence.

Variance learning PER assay collection

Approximately 7500 paint-marked bees were collected between the months of Dec. 2016 and June 2017, paint-marked newly emerged bees were placed into each hive at regular 3-4 week intervals. Age groups were switched approximately every 3-4 weeks, depending upon the dominant foraging paint marked cohort at the time of collection. Eight bees were collected from each hive experience treatment (T and F) each training day and they were divided equally into each training protocol group (see PER assay below).

Calcium Imaging

Bee preparation and PNs staining

Marked bees were captured, briefly cooled on ice and restrained in custom made individual holders suited for calcium imaging (Galizia & Vetter, 2004). After recovery from cooling, the bees were fed with 1.0 M sucrose solution and left undisturbed until staining shortly after. A window was cut in the top of the head capsule, dorsal to the joints of the antennae and rostral to the medial ocellus. The hypopharyngeal glands and trachea near the alpha-lobes (Rybak & Menzel, 1993) were moved and served as visual reference for the staining (Sachse & Galizia, 2002). The tip of a glass electrode coated with fura2-dextran (potassium salt, 10.000 MW, ThermoFisher Scientific) was inserted into both sides of the protocerebrum, dorsolateral to the vertical-lobes, aiming for the lateral antennalprotocerebral tract (l-APT) that contains the axons of uniglomerular PNs (Galizia & Rössler, 2010). A few seconds later, after the dye had dissolved we close the window in the head capsule using the piece of cuticle that had been previously removed. The dye was left to travel along the l-APT tracts until the next day, roughly 10-18 hours. Before imaging, the antennae were fixed pointing toward the front, where odor will be delivered, using a low-temperature melting wax Eicosane. Body movements were prevented by gently compressing the abdomen and thorax with a piece of foam held in place by a piece of tape. The brain was then rinsed with Ringer's solution (130 mM NaCl, 6 mM KCl, 4 mM MgCl2, 5mM CaCl2, 160mM sucrose, 25mM glucose, 10mM HEPES, pH 6.7, 500 mOsmol; all chemicals from Sigma-Aldrich), and glands and tracheae covering the antennal lobes were removed. When necessary we also cut a small

hole between the antennae and mandibles, and then pulled out a small section of a compact structure of muscles, esophagus, and supporting chitin. We did this to put this structure under slight tension and pull it away from the brain to prevent accessory movements in the antennal lobes (Mauelshagen, 1993). Only antennal lobes that presented homogenous staining of all visible glomeruli were used for imaging. Stained bees were then mounted on the microscope and were allowed to recover for 15 minutes before imaging.

Data acquisition and analysis

A Polychrome V (Till-Photonics, Gräfelfing, Germany) was used to emit excitation at two wavelengths alternating between 340 and 380 nm. Imaging data was then collected at 5 Hz using a CCD camera (SensiCamQE, Till-Photonics) mounted on an upright fluorescence microscope (Olympus BX-50WI, Japan) with a 20x objective NA 0.95 (Olympus) using a 505 DRLPXR dichroic mirror and a 515 nm LP emission filter (Till-Photonics). The final spatial resolution of each image was 1376 x 1040 pixels with a pixel side length equaling 2.6 μm. The exposure times during excitation were 8 ms at 340 and 2 ms at 380 nm. The image analysis was done using custom software written in the Interactive Data Language (IDL; Research Systems) using routines created by Giovanni Galizia (University of Konstanz, Germany). Measurements from each animal consisted of a sequence of 50 fluorescence images, obtained at each excitation wavelength (F_i^{340}, F_i^{380}, where subscript *i* is the number of images 1 to 50, and superscript denotes measurements at the excitation wavelengths 340 nm or 380 nm). Calcium responses were calculated as

the ratio $R_i = (F_i^{340}/F_i^{380})$ x 100. We subtracted the background responses (R_b) from these ratios. We calculated R_b by averaging the R_i values 1 second immediately before the odor onset, where $R_b = 1/5 (R_{11} + \ldots + R_{15})$. The resulting relative calcium response measure (ΔR) represents a percentage change from the odor-free reference window (R_{11}–R_{15}). This measures has previously been shown to be directly proportional to changes in intracellular calcium concentration (Galizia & Kimmerle, 2004). Next, we identified glomeruli based upon their morphology and relative position using our own antennal lobe reconstructions and the digital atlases of the honeybee AL (Flanagan & Mercer, 1989a; Cosmas Giovanni Galizia et al., 1999). We also visualized glomeruli using the raw fluorescence images obtained at the 380 nm excitation wavelength. For an additional confirmation of glomeruli locations we created images that represent the degree of correlation between neighboring pixels with a tool provided by Mathias Ditzen (Freie Universitaet Berlin, Germany). Pixels stemming from the same glomerulus are highly correlated over time and pixels from different glomeruli are not. We finally ended up with a common set of 23 glomeruli that could be identified across all animals. All glomeruli corresponded to the dorso-rostral side of the AL innervated by the antennal nerve T1 tract: glomeruli 17, 23, 24, 25, 28, 29, 33, 35, 36, 37, 38, 42, 43, 47, 48, 49, 52, 54, 56, 60, 62; and the T3 tract, 54, 52 (Flanagan and Mercer, 1989; Galizia et al., 1999). The activity for each glomerulus was calculated by averaging mean ΔR activity over a 9x9 px square area that corresponds to approximately a 23.4 x 23.4 μm square which fits within the center of each glomerulus. We then averaged this activity for each glomerulus in each animal over the 1-second odor stimulation period. These values were then used

for the final comparisons of odor-elicited activity across animals and experience treatments.

Aligned vectors for all glomerular responses to all odors or just those responses by a single glomerulus to all odors for animals within each treatment group were then placed into 7 x 7 non-repeating correlation matrices (8 matrices for 1-hexanol tent odor restriction bees, and 120 matrices for freely foraging bees. We next calculated the determinant scores for all matrices (8 1-Hexanol tent bees, 120 freely foraging bees including aligned glomeruli and 23x8 1-Hexanol tent bees, 23x120 freely foraging bees—one set for each measured glomerulus). The observed determinant scores for each treatment group were then compared using the non-parametric Mann-Whitney U test and p-values were corrected using the false-discovery rate procedure when comparing single glomeruli.

Odor stimulation and imaging session

The focus of this analysis was to compare whether a given glomerulus was recruited or not by a given odor, and if this glomerular response profile varied across animals within a given treatment group. For that aim, the glomerular responses were measured for pure odors, mixtures and different concentrations of both. Odors were diluted in mineral oil: 1-hexanol $2x10^{-2}$, $1x10^{-2}$ and $1x10^{-3}$; acetophenone $1x10^{-2}$ and $1x10^{-3}$; mixture1 (1-hexanol $2x10^{-2}$ + acetophenone $1x10^{-2}$); mixture2 (1-hexanol $1x10^{-2}$ + acetophenone $1x10^{-2}$); mixture3 (1-hexanol $1x10^{-3}$ + acetophenone $1x10^{-3}$); 2-octanone $1x10^{-2}$ and $1x10^{-3}$; geraniol $1x10^{-2}$; lemon oil $1x10^{-2}$ and linalool $1x10^{-2}$ M. Ten µl of odor solution were

loaded onto a filter paper strip (0.5 x 4 cm) that was put into a 1 ml glass syringe, which served as an odor cartridge. The odor-delivery device had 14 identical channels, each composed of a three-way solenoid valve (LFAA1200118H; The LEE Company) and an odor cartridge. Valve opening was synchronized with the optical recordings using the acquisition software TILLVisION (Till-Photonics). When the valve opened, the air volume inside the cartridge was delivered (~50 ml/min) into a continuous charcoal filtered air stream (~500 ml/min) which in turn directed the air toward the honey bee head. Thus, the final concentration of odors reaching the honey bee was actually ~1/10 of the concentration in the headspace of the cartridge. Imaging acquisition trials lasted 10 seconds and were separated from each other by one minute. Odor stimulation lasted one second and started three seconds after onset of acquisition. Each odor was tested two times in each animal, making a total of 28 stimulations, including blank trials with mineral oil. Odor order was randomized, with the only restriction being to not use the same odor in two consecutive trials. Behind the honey bee, an exhaust continuously removed air, keeping the arena clean of olfactory stimuli.

Immunocytochemistry

After collection, bees were anesthetized by cooling on ice for a maximum of three minutes, the heads were cut from the abdomen and placed into the 4% paraformaldehyde in phosphate buffer saline (PBS), and the brains were removed and placed in 1 ml of fixative overnight at 4 °C.

Primary antibodies: Affinity-purified goat polyclonal anti-AmOA1 antibodies (21st Century Biochemicals, Inc. Marlborough, MA) were raised against a synthetic peptide acetyl-AMRNDRSPSYSMQVPQQGC-amide, which corresponds to amino acids 547-564 of the honey bee AmOA1 receptor. These antibodies were previously used to study the distribution of the AmOA1 receptor in the honey bee brain (I. Sinakevitch et al., 2011; I. T. Sinakevitch et al., 2013). Monoclonal anti-synapsin (fruit fly synapsin, SYNORF1; clone 3C11) antibodies derived from mice were purchased from DBH (Data Bank HYbridoma). Anti-synapsin binds to protein associated with presynaptic sites of neurons and is largely used for labeling the synaptic neuropil. Rhodamine phalloidin conjugated with TRITC (Tetramethylrhodamine Isothiocyanate (Invitrogen) binds to polymerized F-Actin and was used to label areas of high synaptic turnover and localize areas where growth of (active) synapses occurred.

Secondary antibodies: We visualized AmOA1 using Fab' fragments of donkey anti-goat antibodies conjugated to Cy5 (Invitrogen). Fab' fragments of donkey anti-mouse antibodies conjugated to Alexa 488 were used to visualize anti-synapsin.

Anti-AmOA1 staining procedures on brain sections

Fixed brains were washed in phosphate buffered solution and embedded in agarose (low melting point, Sigma) (8 % w/v). The 80 μm (sagittal, frontal, and horizontal) sections of brains were made using a vibrating blade microtome (Leica VT1000S, Leica Biosystems, Germany) in PBS. Sections were washed (6x20 minutes) in 0.5% of Triton X-100 in PBS (PBSTX) and then were pre-incubated with normal donkey serum (Jackson

ImmunoResearch Laboratories) for 15 minutes. Next, the primary antibodies goat anti-AmOA1 in the PBSTX was added to brain sections at a 1:16 dilution for overnight incubation at room temperature. The next morning, anti-synapsin antibodies were added at 1:1600 for six hours incubation at room temperature and, after washing an additional 6 times with PBSTX (6x20 minutes), sections were incubated overnight at room temperature with secondary antibodies (donkey anti-mouse antibodies conjugated with Alexa 488 at 1:270, donkey anti-goat antibodies conjugated with Cy5 at 1:200) and PhalloidinTRITC. Sections were then washed in PBS (6x10 minutes) and embedded on slides in mounting medium.

Anti-synapsin staining procedures on whole-mount brains

After fixation procedures described above, brains of free foraging honey bees (*Apis mellifera carnica*) were washed in PBSTX (6x20minutes). Following washes, brains were pre-incubated with normal donkey serum for 15 minutes and incubated for three nights at room temperature with anti-synapsin at 1:800 in PBSTX solution. The following day, brains were washed again in PBSTX (6x20minutes) and incubated for three nights at room temperature with donkey anti-mouse antibodies conjugated with Alexa 488 at 1:270 and then with Phalloidin at 1:160 in PBSTX overnight. After secondary staining, brains were washed with 4% paraformaldehyde fixative for 10 minutes and then put through a dehydration protocol using increasing steps of ethyl alcohol. Following full dehydration and three washes in 100% ethanol, brains were washed in methyl salicylate until tissue had cleared. They were then mounted on slides in methyl salicylate.

Confocal Image collection and processing

Images were collected using a Leica TCS SP5 confocal laser scanning microscope (Leica, Bensheim, Germany) with a Leica HCX PLAPO CS 40x oil-immersion objective (numerical aperture: 1.25) using the appropriate filter and laser setting for each florescent molecule used (see sections above). Image stacks were collected using 2 μm optical sections and a total volume of approximately 20 μm. Image stacks were then flattened using maximum intensity functions in the Leica software. Image size, intensity, and resolution were adjusted using Adobe Photoshop CC.

3D dorsal part of antennal lobe reconstruction

To identify the glomeruli number of sectioned preparations we used AVIZO software (Thermo Scientific, FEI) to make a 3D reconstruction of the antennal lobe from a whole mount preparation. We then identified glomeruli (comparison with honey bee atlas reference, and Galizia et al. 1999), and made virtual (sagittal) sections of this reconstruction.

Tiff files of whole mount brain confocal scans were taken every 10 μm throughout the honey bee antennal lobe left and right. These files were imported into AVIZO software, and the voxel dimension outputs of each image stack were imported to provide correct dimensions (Thermo Scientific, FEI). Stacked images were then converted to a single channel, so that only anti-synapsin staining was present. Using the image segmentation function, we identified individual glomeruli on the dorsal surface of the left and right antennal lobes. Glomeruli were individually labeled by hand throughout the image stacks.

Due to limitations of the Leica SP5 objective working distance and resolution when imaging through large volumes of tissue, we were only able to scan the frontal half of the antennal lobe. A volume rendering of these labels was created, and exported into Photoshop CC. In Photoshop, we created a manipulatable 3D image using the 3D volume function.

Proboscis extension response variance learning

Eight harnessed bees from each odor experience treatment group (F and T) were collected and presented with an odor mixture paired with sucrose at a 10-minute inter-trial interval using an odor delivery system each training day (B. H. Smith & Burden, 2014; Wright & Smith, 2004). Stimuli were delivered by passing air through a glass cartridge containing 20 µl of an odor mixture on a small strip of filter paper. Each odor cartridge was used for no more than six presentations.

Odor blends of three monomolecular odorants were used and mixed with hexane as a solvent. Two classifications of odor mixtures were produced. The first blend was comprised of: phenylacetaldehyde, nonanal, α-farnesene. The second blend consisted of acetophenone, geraniol and 2-octanone.

As in Wright and Smith (2004), in the variable mixtures, one of the three odors was presented consistently over all trials at a constant concentration. Other odors were varied from trial to trial, using either a high (H: 2.0M) or low (L: 0.0002M) concentration

(Wright & Smith, 2004). Four mixtures were prepared for each odor set as follows: LLH, LHL, LLL, and LHH. With the constant odor, phenylacetaldehyde or acetophenone were presented consistently at the low concentration for each blend. Ratios of each odor mixture were then confirmed using GC/MS. It is important to note that the means of concentrations across all odor presentations were equal. Each subject was presented the above odor mixtures over 16 trials in a pseudorandomized order, with each odor being presented four times over the process of training.

An additional group of bees was trained in the same way as above, however, the odor mixture ratios were held constant across the entire training process. Bees from both the natural free experienced group and the tent-restricted odor experience group were trained under either the varied or constant mixture conditions. At the end of conditioning, the bees were placed in a humidified container. After two hours, the subjects were tested for a PER response to the individual component odors that comprised the training odor mixtures, separately, at the low concentration (0.0002M). Responses to each odor component were presented without any sucrose reinforcement, and PERs were recorded (Wright & Smith, 2004). Test odors were presented in a randomized order.

We analyzed responses for acquisition using Generalized linear model (GLM), and for recall using Generalized Linear Mixed Model (GLMM) to allow bee identity to be included as a random factor. Finally, we compared differences in recall responses

between the variable and constant acquisition protocols between treatment groups using the non-parametric Mann-Whitney U test.

CHAPTER 4

BEHAVIORAL AND NEURAL RESPONSES TO THE NATURAL BRASSICA RAPA FLORAL ODOR AND ITS COMPONENTS IN HONEY BEES

INTRODUCTION

Natural floral odors generally contain several chemical components, and these mixtures are variable across individual inflorescences (Knauer & Schiestl, 2015; Levin et al., 2003; Raguso, 2008). However, pollinators readily learn single monomolecular volatiles associated with floral resources (Knauer & Schiestl, 2015; B. H. Smith & Burden, 2014; Wright & Schiestl, 2009). In the present work we ask how are complex bouquets processed by the olfactory system of honey bees and do the nervous system of bees show some sensory bias towards mixtures? Perhaps there are additional sensory selection pressures imposed by pollinators that could explain the discrepancy between the signals used by flowers and what chemical odorants bees may most directly be associating with resources. I am proposing 3 potential hypotheses centered on the olfactory systems of bees to explain this observation.

Hypothesis 1: Complex natural mixtures are more robustly processed by the olfactory systems of animal pollinators, enabling pollinators to recognize the same signal even if some components might be missing in the floral bouquet. Hypothesis 2: These mixtures provide a greater range of variation and have the potential to signal a greater amount of information to potential pollinators aside from simply presence or absence. This

information could provide selective advantages to both the flowering plants and the pollinators, if the pollinators make use of this information. Hypothesis 3: Pollinators are inherently more attracted to more complex odorants. This would put a current selection pressure on flowering plants to display complex stimuli to attract these pollinators. In the present study, we measured how a pollinator behaviorally and physiologically responded to a natural floral mixture compared to its constituent components. We tested if the mixture is innately more attractive to the pollinator (hypothesis 3) and if the physiological signaling dynamics of mixtures are different from those of single components in the central nervous system (hypotheses 1 & 2).

We used floral volatiles of *Brassica rapa* as the stimulus. *B. rapa* is an important agricultural species and has a well-characterized evolutionary history as well as known volatile chemicals associated with both its leaves and flowers (Knauer & Schiestl, 2015; Miyazawa, Nishiguchi, & Yamafuji, 2005). *Brassica rapa* typically has 12-20 primary components that make up its floral odorant bouquet (Knauer & Schiestl, 2015). *B. rapa*'s volatiles act as signals of nectar and pollen to bumble bee and honey bee pollinators, and the volatiles do not correlate with defense compound production (Knauer & Schiestl, 2015; Schiestl, 2014).

We used the honey bee, *Apis mellifera,* to measure responses to *B. rapa*'s volatiles. Honey bees are an agriculturally important generalist pollinator for many plant species, including *Brassica rapa* (Aizen & Harder, 2009; Brittain, Williams, Kremen, & Klein,

2013). Both the behaviors and the olfactory system of honey bees are well characterized (Cosmas Giovanni Galizia et al., 1999; Galizia & Menzel, 2000; Hammer & Menzel, 1998; Menzel, 1985; Menzel & Erber, 1978). Honey bees readily respond to volatile stimuli and can be trained to forage at diverse flower species and artificial odorant stimuli in free flight (Menzel et al., 1993). Honey bees also readily behave in a restrained setting and can be classically conditioned using the proboscis extension response (PER) to associate floral volatiles with sucrose (Bertram Gerber et al., 2009; Guerrieri et al., 2005; B. H. Smith & Burden, 2014). It is also possible to measure neural responses at several levels of processing in the nervous system of honey bees. These include measurement of the local field responses of peripheral odorant receptors using electroantennograms (EAG) (Bhagavan & Smith, 1997; De Jong & Pham-Delegue, 1991; Henning & Teuber, 1992) and—more centrally in the primary olfactory neuropil—the antennal lobes (AL), using calcium imaging and electrophysiology (Brill et al., 2013; Locatelli et al., 2013; Sachse & Galizia, 2002; Strube-Bloss et al., 2011).

Foraging honey bees show preferences for novel flowers that have similar volatiles to learned scent cues, and they will reject scents that are too dissimilar from their target (Guerrieri et al., 2005; Wright & Schiestl, 2009). In *Brassica rapa*, one of the floral components, phenyl acetaldehyde, statistically correlates with presence of pollen and nectar resources (Knauer & Schiestl, 2015). A related Hymenopteran, the bumble bee (*Bombus terrestris*), will develop a preference for phenyl acetaldehyde when that odor is tested alone when they are foraging on *B. rapa* (Knauer & Schiestl, 2015). Additionally,

honey bees can learn and discriminate *Brassica rapa*'s floral mixture bouquet from the bouquets of other species (Wright, Skinner, & Smith, 2002).

However, the preference for phenyl acetaldehyde is perplexing, as there are several other components in the *B. rapa* odor bouquet. Honey bees are capable of detecting almost all of the bouquet components, as they show electrophysiological responses to all of the floral components of the *Brassica* floral volatiles (EAG, *personal comm.* Ghaninia). To test the above hypotheses, we first evaluated how foragers behaviorally responded to the entire mixture and to its components using an antennal movement assay. Then, we correlated these responses to how the central nervous system processed the components and the more complex natural floral mixture.

RESULTS

Behavior

Bees show reflexive antennal responses to odors and mechanosensory stimuli (Suzuki 1975), and these antennal movements shift with odor experience (Cholé et al., 2016; Lei et al. *in prep*). These responses should be more sensitive to measure differences in odorant responses than the standard proboscis extension protocol. Therefore, we measured the antennal responses of honey bee foragers to the *Brassica rapa* floral odor mixture and, separately, to its constituent components. We presented a total of 16 odorant stimuli once each, one at a time to each bee using a randomized sequence. Twelve presentations were with the *pure* components of the *B. rapa* mixture presented at

statistically equivalent EAG intensities (ANOVA, df=6, p=0.16). An additional two presentations were with two different concentrations of the full *Brassica* mixture (one high concentration and a second low mixture that was 10 times less concentrated). Each component in both of the mixtures was at an equivalent $1/12^{th}$ molecular concentration. Finally, two blank air stimulations were presented, one at the beginning of the presentations and one half way through the full series (**Table 4.1**). The low mixture concentration showed equivalent electrical stimulation to the antenna of the honey bee (electroantennogram, EAG) as the 12 individual *Brassica* components and the high mixture was more concentrated, but also not statistically different from the mean EAG intensity for each of the individual components (*personal comm.* M. Ghaninia).

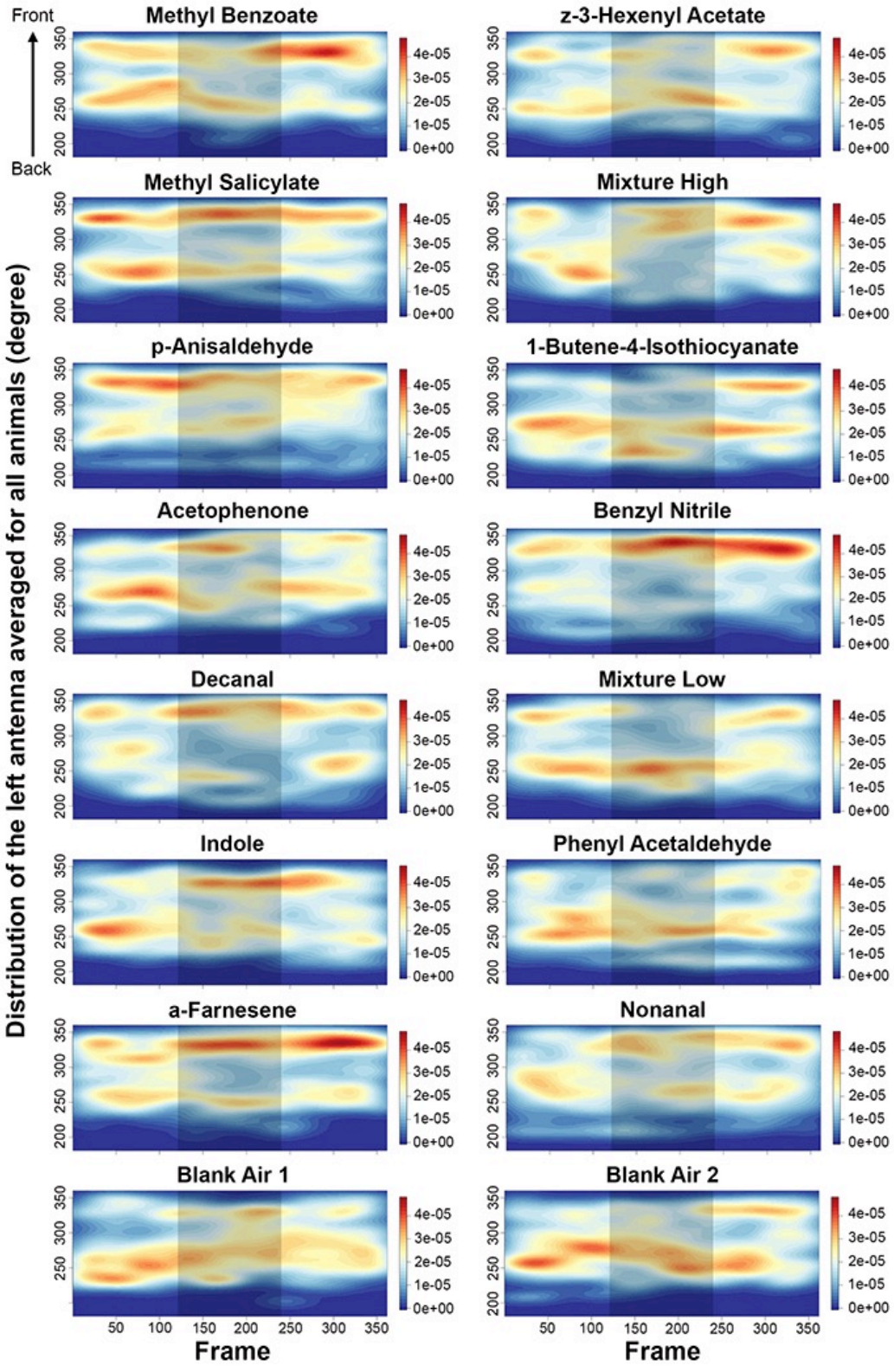

Front
Back
Methyl Benzoate
z-3-Hexenyl Acetate
Methyl Salicylate
Mixture High
p-Anisaldehyde
1-Butene-4-Isothiocyanate
Acetophenone
Benzyl Nitrile
Decanal
Mixture Low
Indole
Phenyl Acetaldehyde
a-Farnesene
Nonanal
Blank Air 1
Blank Air 2
Distribution of the left antenna averaged for all animals (degree)
Frame
Frame
200
250
300
350
50
100
150
200
250
300
350
0e+00
1e-05
2e-05
3e-05
4e-05

Figure 4.1. Density heat map distributions of the angular position of the tip of the left antenna relative to the center of each bee's head, mean distributions across 20 animals, x-axis is centered on the odor stimulus onset. Each plot represents 12 s of time, 4 s before the odorant stimulus (standardized frames 1-120), 4 s during the odorant stimulus (standardized frames 121-240; shaded box), and 4 s after the odorant stimulus (standardized frames 241-360). Each odorant stimulus is given its own panel and labeled above each plot. The top of each plot (360) is the direction closest to the odor source (and the bee's mandibles) and the bottom (180) is furthest away from the odor source. The color-density scale is the same across all plots, and was automatically generated using the two-dimensional kernel density estimating function in the Mass package in R using modified code from Birgiolas, Jernigan, Gerkin, Smith, and Crook (2017). Stimuli are arranged from left to right and top to bottom by their mean glomerular activation averaged across 11 animals measured using calcium imaging (see Figure 4.3B). See Table 4.1 for concentration information.

Table 4.1. Odor stimuli and prepared concentrations in hexane solvent.

Odor	Concentration (pg/L)
α-Farnesene	$1x10^{14}$
Acetophenone	$1x10^{12}$
Benzyl Nitrile	$1x10^{14}$
Decanal	$1x10^{12}$
Indole	$1x10^{14}$
Methyl Benzoate	$1x10^{14}$
Methyl Salicylate	$1x10^{14}$
Mixture Low	$1x10^{12}$
Mixture High	$1x10^{13}$
Nonanal	$1x10^{12}$
p-Anisaldehyde	$1x10^{13}$
Phenyl Acetaldehyde	$1x10^{13}$
z-3-Hexenyl Acetate	$1x10^{14}$

Each odorant stimulation video was then processed using custom software: SwarmSight (Birgiolas, Jernigan, Gerkin, et al., 2017), to extract antennal positions and angles relative to the head in each frame of the videos. We analyzed the angular position of the left antennae relative the position of the head for each of the 16 stimuli, averaged across all 20 bees (**Figure 4.1**). Each of the odors shows a unique antennal response pattern when treating bee identity as a random factor (**Table 4.2** GLMM; Left Angle~Odor*Odor time; Bee identity as a random factor to remove unique idiosyncratic movements, df=585,981; $p<0.05$).

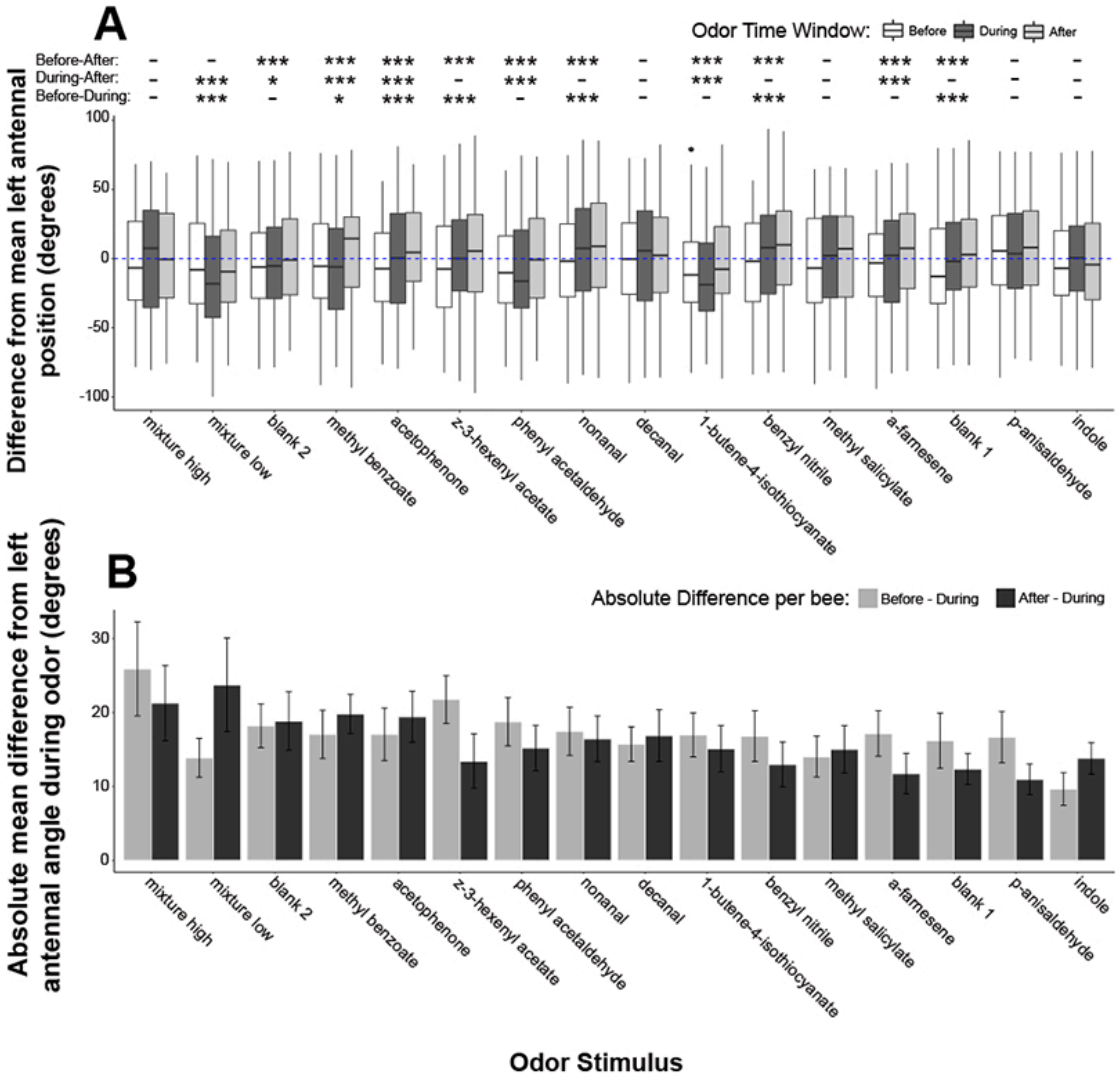


Figure 4.2. The difference from the mean resting antennal position before-, during-, and after-odor stimulus is presented as a measure for the intensity of the antennal response. **A**. The pooled left antennal angle changes from the mean resting position 4s before the odor (white), 4s during the odor stimulus (dark gray), and 4s after the odor stimulus (light gray). **B**. The absolute mean angle difference before (light gray) and after (dark gray) odor stimulation minus the angle mean during odor stimulation, averaged per bee. Odorants arranged from left to right by averaged difference from before and after odor

stimulation minus during odor stimulation. See Table 4.1 for concentration information. Statistics: A. ANOVA difference from mean ~ Odor*Odor time, df= 30, Tukey HSD post hoc test, *** $p<0.001$, ** $p<0.01$, * $p<0.05$, - $p>0.05$. B. T-test that differences were > 0 with false discovery rate corrected p-value, df= 10-19, all $p<0.01$, ANOVA, difference~odor, df= 15, no response differences significantly different from one another.

While the behavioral responses are statistically unique for each odor (**Figure 4.1**, **Table 4.2**), we wanted to get a measure of the degree of response intensity for each stimulus. To do this, we subtracted the antennal angle in each video frame from the mean angle for each bee video when no odor was present (over the full video length) to account for movement response differences between bees and for any alterations in the mean antennal resting position throughout the 1.5 hr protocol.

Table 4.2. Generalized Linear Mixed Model output of Left antennal angle with interacting predictor of Odor by Odor time and a non-interacting frame factor and with bee identity as a random factor (Left antennal angle ~ Odor*Odor time + Frame 1|~Bee Identity).

Factor	Value	DF	t-value	p-value
Intercept	283.729	585981	128.677	0.0000
α-Farnesene	7.358	585981	26.589	0.0000
Acetophenone	1.532	585981	5.616	0.0000
Benzyl Nitrile	5.689	585981	21.098	0.0000
Blank Air 1	-4.809	585981	-17.598	0.0000
Blank Air 2	-2.019	585981	-7.487	0.0000
Decanal	1.336	585981	4.954	0.0000
Indole	-1.913	585981	-7.092	0.0000
Methyl Benzoate	7.802	585981	28.926	0.0000
Methyl Salicylate	2.503	585981	9.176	0.0000
Mixture Low	1.206	585981	4.471	0.0000
Mixture High	0.210	585981	0.661	0.5086
Nonanal	-0.081	585981	-0.301	0.7631
p-Anisaldehyde	1.017	585981	3.771	0.0002
Phenyl Acetaldehyde	2.389	585981	8.856	0.0000
z-3-Hexenyl Acetate	2.993	585981	11.099	0.0000
During-Odor	-11.166	585981	-14.731	0.0000
Frame	0.005	585981	60.551	0.0000
α-Farnesene:During-Odor	11.116	585981	10.108	0.0000
Acetophenone:During-Odor	12.998	585981	11.987	0.0000
Benzyl Nitrile:During-Odor	16.543	585981	15.453	0.0000
Blank Air 1:During-Odor	13.876	585981	12.795	0.0000
Blank Air 2:During-Odor	10.679	585981	9.976	0.0000
Decanal:During-Odor	14.336	585981	13.391	0.0000
Indole:During-Odor	13.187	585981	12.318	0.0000
Methyl Benzoate:During-Odor	6.965	585981	6.506	0.0000
Methyl Salicylate:During-Odor	14.076	585981	12.981	0.0000
Mixture Low:During-Odor	1.903	585981	1.778	0.0754
Mixture High:During-Odor	14.683	585981	11.564	0.0000
Nonanal:During-Odor	18.343	585981	17.134	0.0000
p-Anisaldehyde:During-Odor	18.216	585981	17.016	0.0000
Phenyl Acetaldehyde:During-Odor	2.634	585981	2.461	0.0139
z-3-Hexenyl Acetate:During-Odor	12.724	585981	11.886	0.0000

Most odors showed a significant shift in the mean angle difference for the left antenna with mixture low, methyl benzoate, acetophenone, z-3-hexenyl acetate, nonanal, benzyl nitrile and blank 1 showing a significant shift from before- to during-odor stimulation (**Figure 4.2A**). The bees generally pulled their antennae away from the low mixture, while the rest of these significant shifts showed movement toward the odors (**Figure 4.2A**: methyl benzoate, acetophenone, z-3-hexenyl acetate, nonanal, benzyl nitrile, and blank 1). Additionally, blank 2, methyl benzoate, acetophenone, z-3-hexenyl acetate, phenyl acetaldehyde, nonanal, 1-butene-4-isothiocyanate, benzyl nitrile, a-farnesene, and blank 1 have a significant shift in angle position from before- to after-odor stimulation, potentially suggesting some post-stimulation effects (**Figure 4.2A**). In all of these significant cases, this shift from before- to after-odor was toward the odor direction. This is likely similar to increased sniffing behavior observed in mice after odor detection (**Figure 4.2A**, (Wesson, Donahou, Johnson, & Wachowiak, 2008; Wesson, Verhagen, & Wachowiak, 2009)). Mixture low, blank 2, methyl benzoate, acteophenone, phenyl acetaldehyde, 1-butene-4-isothiocyanate, and a-farnesene have a significant angle distribution shift from during-odor stimulation to after-odor stimulation (**Figure 4.2A**).

This change in response after odor removal could reflect a transition to increased search in the direction of the odor as mentioned above; all of these significant shifts were toward the direction of odor delivery or to re-explore the space that was ignored during odor responses as they held antenna back during odor stimulation (**Figure 4.2A**). In the case of the low mixture, phenyl acetaldehyde, and 1-buten-4-isothiocyanate, there was a trend to

pull the antenna away during the odor stimulation, with a subsequent rebound after the odor was removed to move the antennae back toward the odor delivery direction. Such a response could be either the enhanced "sniffing" behavior suggested for the other odorants or simply a "re-scanning" of the space in front of the bee that was ignored during the odor delivery. The low mixture is the only odor in this last group that does not also show a difference between before- and after-odor stimulation (**Figure 4.2A**); this suggests that, perhaps due to a more uniform response to the low mixture than the other odors, most or all bees pulled antenna back. Bees showed more variable responses to other odors— i.e., some bees may pull antennae away while others move their antennae toward the odorant. This directional variation was quite strong for the high odor mixture responses and caused the general directional shifts to be non-significant (**Figure 4.2A**).

To control for this directional variation between bees, we used the absolute difference of these mean response measures for both the before- and the after-odor stimulus periods minus the during-odor stimulation periods for each bee and odorant stimulus. All of these shifts were significantly different from zero (**Figure 4.2B**, t-test with false discovery rate corrected p-values, df= 30, $p<0.01$). Additionally, the high and low mixture stimuli had the largest differences in these measures, however, this trend was not significantly different from the other odors (**Figure 4.2B**, ANOVA, difference~odor, df= 15, $p>0.05$). Of note, the low intensity mixture, despite all of the components being $1/12^{th}$ the concentration of those presented as single components, still showed some of the strongest antennal response shifts (**Figure 4.2**). This difference provided initial support for

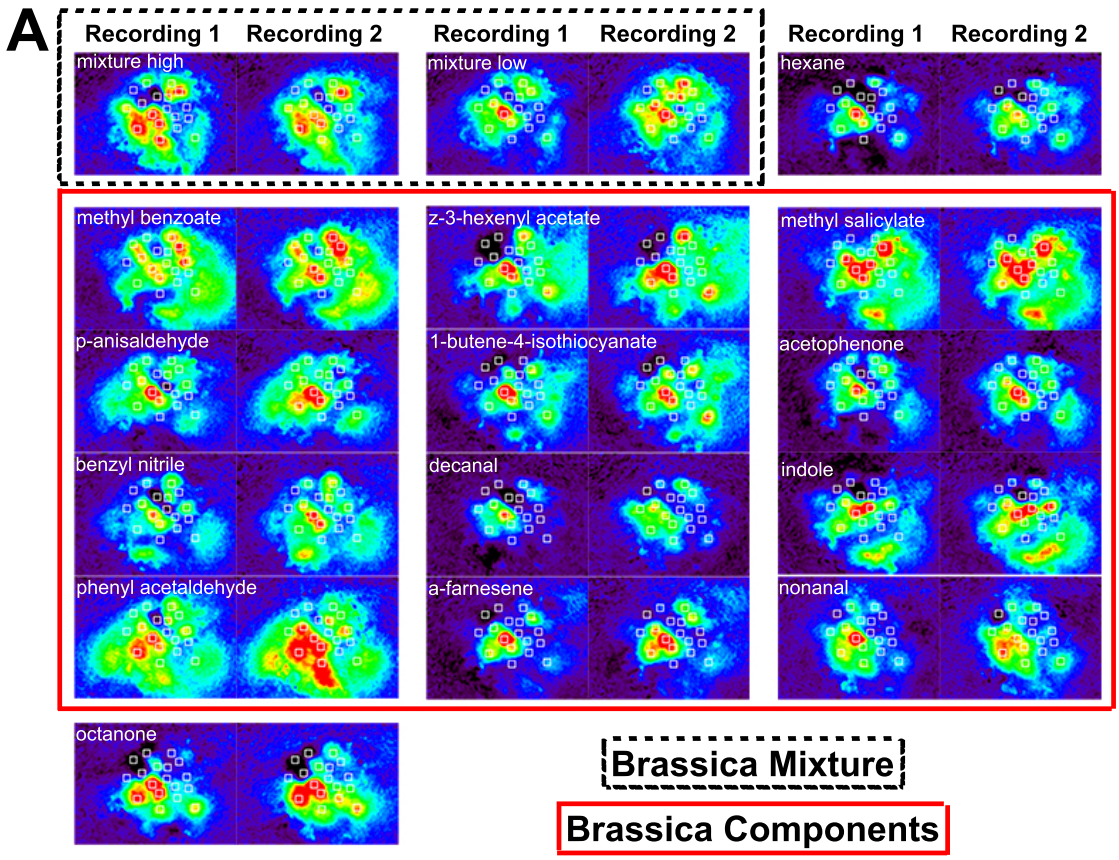
hypothesis 3 in that the mixtures might be qualitatively more salient for generating behavioral responses.

Physiology

We next evaluated the central nervous system responses to the *Brassica rapa* floral mixture and its constituent components. We used a Fura-2 dextran dye injection to the lateral-antennoprotocerebral (l-APT) projection neuron tract near the vertical lobe of the mushroom bodies; this helped to visualize the projection neuron responses in glomeruli that lie in the rostro-dorsal part (primarily T1) of the antennal lobe (Sachse & Galizia, 2002). We presented each of the 12 *B. rapa* mixture components and the 2 mixture intensities at the same intensity as above, as well as the hexane solvent and octanone as an external odorant control (**Figure 4.3A**). We recorded 18 identified glomeruli in 11 animals for the 16 odorant stimuli, twice per animal (**Figure 4.3A**-Recording 1 and Recording 2). The recordings for each stimulation lasted 10 seconds. The odor was delivered 3 seconds after recording onset and odor stimulation lasted for 1 second.

There was significant variation in the mean activation of each odor across the 18 glomeruli despite the fact that the concentrations used give rise to equivalent EAG intensities (**Figure 4.3**). Methyl benzoate was the most stimulating to the 18 measured glomeruli across all animals followed by z-3-hexenyl acetate, methyl salicylate, and the high concentration mixture (**Figure 4.3B**). The highest stimulating odors generally activated 3 or more glomeruli to a high degree, where as those at the lower end generally

activated 2 or fewer glomeruli (**Figure 4. 3B**). Both mixture intensities fell in the middle level of glomerular activation (**Figure 4. 3**).


A
Recording 1
Recording 2
Recording 1
Recording 2
Recording 1
Recording 2
mixture high
mixture low
hexane
methyl benzoate
z-3-hexenyl acetate
methyl salicylate
p-anisaldehyde
1-butene-4-isothiocyanate
acetophenone
benzyl nitrile
decanal
indole
phenyl acetaldehyde
a-farnesene
nonanal
octanone
Brassica Mixture
Brassica Components

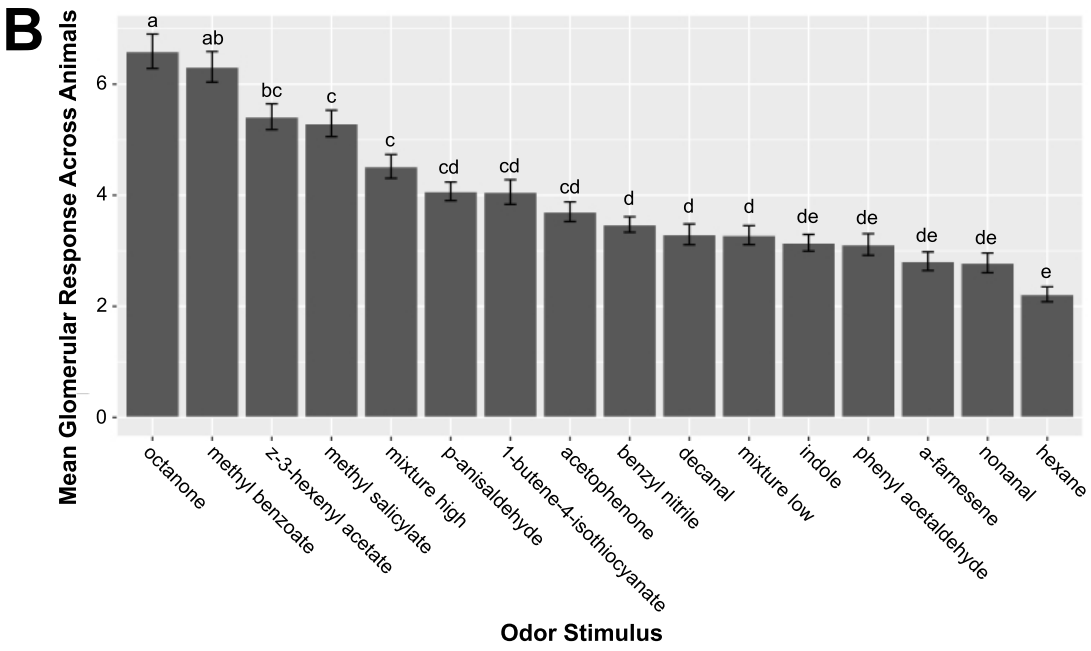

B
Mean Glomerular Response Across Animals
0
2
4
6
a
ab
bc
c
c
cd
cd
cd
d
d
d
de
de
de
de
e
octanone
methyl benzoate
z-3-hexenyl acetate
methyl salicylate
mixture high
p-anisaldehyde
1-butene-4-isothiocyanate
acetophenone
benzyl nitrile
decanal
mixture low
indole
phenyl acetaldehyde
a-farnesene
nonanal
hexane
Odor Stimulus

Figure 4.3. Glomerular responses to *Brassica rapa* and its constituent components. **A**. Example recording 1 and 2 from one animal. **B**. The mean glomerular responses across all glomeruli averaged from all 11 animals. Statistics: ANOVA mean response ~ Odor, df= 15, Tukey HSD post-hoc analysis letters denote significant difference $p<0.05$. Odorants arranged from left to right by mean glomerular activation averaged across 11 animals. *Brassica* mixtures highlighted in black dashed line, *Brassica* components highlighted in red lines. See Table 4.1 for concentration information.

We next measured the glomerular pattern consistency across odors. To do this we created all possible 7x7 (bee x bee) correlation matrices (120 possible matrices for octanone, 8 possible matrices for low mixture, and 330 possible matrices for all other odors). We then calculated the determinant score for each matrix as a measure for glomerular pattern consistency between animals for each odorant (**Figure 4.4**). The least consistent glomerular odor pattern between animals was phenyl acetaldehyde—i.e. the identity and strength of the glomeruli activation was the most variable between bees for phenyl acetaldehyde (**Figure 4.4**). The two mixture concentrations fell in the middle with regards to glomerular odor pattern consistency between animals (**Figure 4.4**). Overall, glomerular odor pattern consistencies were not clearly correlated with mean glomerular activation (**Figure 4.3B**).

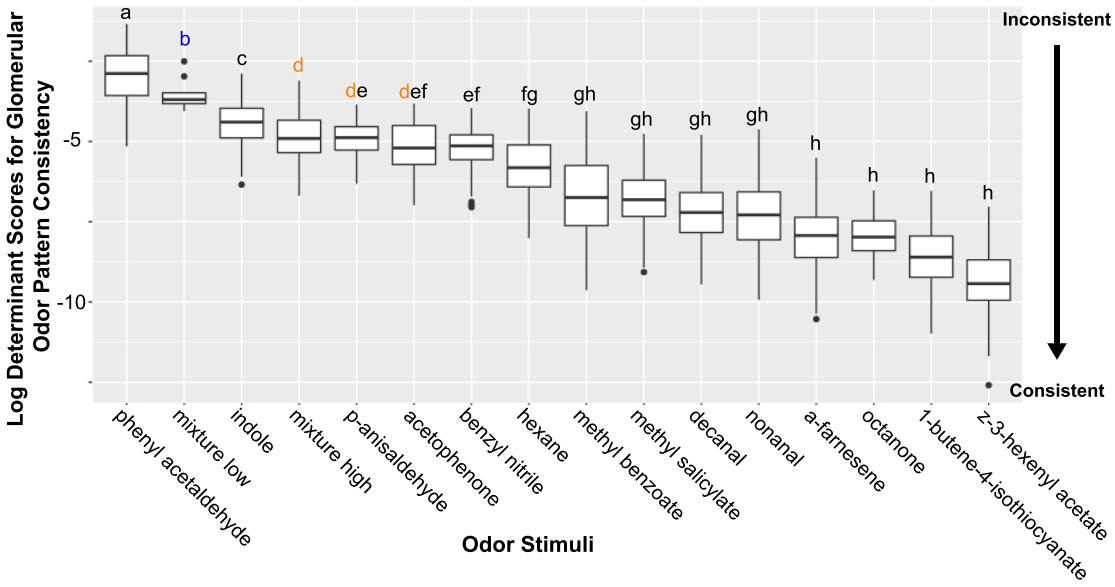


Figure 4.4. The glomerular odor pattern consistency between animals. The log transformed determinant score measure for all possible non-repeating 7x7 (bee x bee) correlation matrices comparing 11 animals: 120 possible unique matrices for octanone, 8 possible unique matrices for the low concentration mixture, and 330 possible matrices for all other odors—some animals did not have measurements for all odors. The determinant score is the inverse of a correlation measure; a high determinant score indicates a low between-animal glomerular pattern consistency to an odor, and vice versa. Statistics: ANOVA, determinant score ~ odor, df= 15, Tukey HSD post hoc test, letters denote statistical differences $p<0.05$. Colors denote statistically the same as low mixture (blue) and high mixture (orange).

The between-animal glomerular pattern is no more consistent for the mixtures than any of the single components, however, it is possible it is more consistently represented within each animal between stimulations. To test this, we compared the response correlations

with each glomerulus within each animal between recording 1 and recording 2 (**Figure 4.5**). We compared these correlations among the 16 odorant stimuli and found that the high concentration mixture showed the highest within-glomerulus response consistency; however, this was not significantly different from the other top 8 odorants (**Figure 4.5**, ANOVA, correlations ~ odor, df=15, Tukey HSD post hoc test). This finding provides some support for hypothesis 1 in that the mixture might be more robustly processed and signaled more reliably among glomeruli within each animal, allowing the odor to be recognized more easily between presentations. Based upon this finding, we would predict that animal pollinators might more readily recognize and recall mixtures over single components, but this requires further testing.

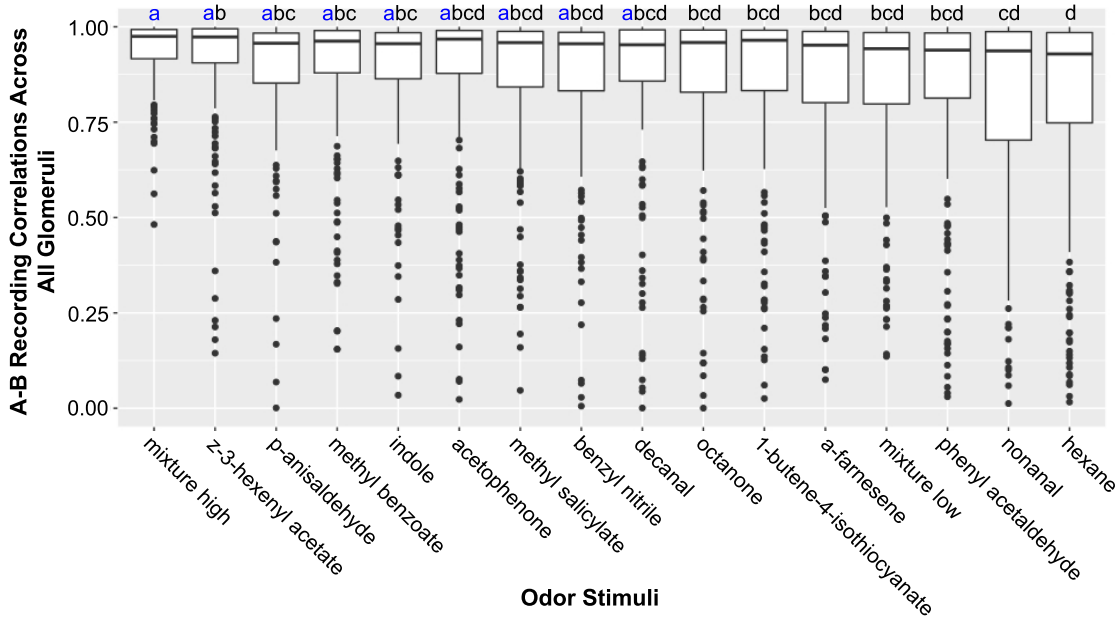


Figure 4.5. The correlations of within-glomerulus recordings 1 and 2 versus odorant stimuli. Odorants arrayed from left to right from most to least correlated. Statistics:

ANOVA, correlations ~ odor, df=15, Tukey HSD post hoc test letters denote significance $p<0.05$. Blue color denotes statistically the same as high concentration mixture.

DISCUSSION

These data provide additional support that pollinators tend to be both behaviorally more stimulated by mixtures and thus potentially more attracted to mixtures (hypothesis 3) and that mixtures are more consistently represented between presentations in the central nervous system of honey bees (hypothesis 1). Both of these would provide a strong selection bias on flowering plants to produce mixtures to attract and signal to pollinators.

In the 18 glomeruli we measured and identified, we did not see evidence that the glomerular pattern for mixtures is more complex than responses to single odorants, in the 18 glomeruli we were able to measure and identify in our preparations. The overall mean glomerular activation for mixtures was no greater than what was observed for many of the single components. It is possible that this would change if we could measure glomerular responses throughout the entire antennal lobe.

We measured the antennal responses to odorants based upon prior work in our lab (Birgiolas, Jernigan, Gerkin, et al., 2017). We chose to use antennal responses because they could potentially be more revealing of subtle response difference that would be more difficult to elucidate using standard PER training, and these assays can be done relatively quickly with a very large panel of odors. There is precedence for this idea, and the use of

antennal movement to measure behavioral responses to odors is not new. Antennal movements are not random and change with both associative and non-associative conditioning (Cholé et al., 2015; Kisch & Erber, 1999; Suzuki, 1975). Suzuki (1975) showed that odor stimulation to the antennae of the honey bee induced increase movement by the antennal muscles. Kisch and Erber (1999) were able to conduct operant conditioning of the antennal movements of honey bees using several different paradigms. More recently Cholé et al. (2015) were showed changes in antennal movement to odors that were classically conditioned with sucrose.

In our study we found that antennal movements to odor are somewhat unique for every individual. To attempt to control for this idiosyncrasy between animals we subtracted the angle in each video frame from the mean angle of each bee video when no odor was present. This measure provides an assessment of degree of response that can be standardized across animals regardless of the known movement differences between animals. The final response intensity measure we used took the absolute difference of these measures for the before and the after periods minus the during stimulation period, because some bees tended to move their antennae towards odors while others moved them away. We do not yet know what the source of these differences were. It could relate to the experiences of the bees while freely foraging or to the motivation state to respond to odors (e.g., experienced versus un-experienced forager, guard bees versus forager). However these collection methods are very common and are present in almost all learning and physiological studies using honey bees (Galizia & Menzel, 2000).

In general, the behavioral intensity measures, to some degree, tracks with the odors that show the steepest EAG dose response curves. We presented odors at intensities that gave equivalent EAG responses. However there could be intensity differences we could not statistically distinguish using standard EAG methods. There are also a few notable exceptions. For example, methyl benzoate and z-3-hexenyl acetate show a much stronger behavioral response than the EAG dose response curves might suggest. This is also the case for the mean glomerular activation, however the mean glomerular activation and the behavioral response intensities do not correlate. We also observed a high response to the second blank air trial, unlike the first blank air trial. This could be a sensitization effect of the bee expecting an odor. Sucrose is known to sensitize response to odors (Hammer et al., 1994), and it is possible odors might sensitize antennae to mechanosensory stimulation. However that remains to be tested. Randomizing the presentation of odors, as we did, should control for any sensitization effects.

Overall, there is a clear trend that both the high and low intensity mixtures cause the largest shift in responses during odor presence. This result suggests that mixtures are more behaviorally stimulating to bees than their mono-molecular components. We could follow this up with a series of binary choice assays of novel mixtures versus single components in a foraging task to test if this selection bias exists in a more natural foraging environment. This high behavioral response intensity to the mixtures is correlated with a high within-glomerulus response consistency between presentations.

Because of this trial-to-trial stability we would predict, in further experiments, that mixtures might be more easily recognized between exposures; making them more easily learned and recalled by the central nervous system of bees.

It was interesting that the low concentration mixture was not as reliably represented within glomeruli between presentations. There is likely some dose-dependent effect, in that the high intensity mixtures may also be more clearly represented in the central nervous system. It would be worth investigating if this is the case. Perhaps the additive effects of glomerular activation with concentration are lessened with a larger number of constituent components, allowing for a clearer mixture representation along a larger concentration gradient, which is another possible hypothesis to support the selection of large component mixture signals in flowers.

In conclusion, we observed strong trends that odor signals composed of complex mixtures could be selected over single – i.e. simpler - odorant components. These findings support hypothesis 3, in that they are both more behaviorally stimulating, and hypothesis 1, in which mixtures are more reliably signaled in the pollinators' central nervous systems.

METHODS

Honey bee collection

Returning bees without pollen loads were collected at the nest entrance from a hive on Arizona State University campus, Tempe, AZ, USA. Honey bees were placed in a small glass vial that was chilled until bees were immobilized. Of note, the bees in this location are highly unlikely to have prior experience with the full *Brassica rapa* odor bouquet as this species is not cultivated in Arizona.

Behavioral harnessing

The honey bees were then placed in plastic harnesses and secured with a small piece of duct tape. Each tube had a small opening at the bottom to allow for airflow to the abdomen. The top of the tube was then covered with a light-colored masking tape and the back of the bee's head was fixed in place with a small amount of melted wax (Birgiolas, Jernigan, Gerkin, et al., 2017; B. H. Smith & Burden, 2014). Bees were fed 30% (w/w) sucrose allowed to rest in the harness for approximately 40 min. We selected bees that showed active antennal movements after this procedure to ensure that the bees' antennae were not damaged during this procedure and that the bees were healthy and alert.

Odor preparation

Odors were diluted in hexane to the concentrations listed in Table 4.1. We used GC/MS to later confirm concentrations were accurate in mixtures. We pipetted 20 µl of odor were placed onto a small piece of filter paper—we allowed a few seconds for hexane to

evaporate off of the filter paper and then placed the odor-laden filter paper inside of a glass cartridge which was then sealed with Parafilm. Glass odor cartridges were used twice at most to ensure stimulus content was always delivered at equivalent (undiminished) concentrations across trials.

Behavioral protocol

Each harnessed bee was placed into an odor delivery system, in which constant humidified air was delivered to the bee. An odor stimulus could be injected into the air stream. Constant humidified air was delivered at approximately 124 ml/min and with the added odor stimulus the flow rate increased to approximately 158 ml/min. A vacuum behind the bee pulled away excess odorants after delivery. A web camera was placed above the bee to record antennal responses, and an LED light was activated during odor presentation. Each video was recorded at 30 frames per second and lasted between 50-65 s in duration. The odors were each presented once to each bee in a randomized order. We used a 5-minute inter-stimulus interval between odor presentations to minimize effects of sensory adaptation. We also presented two blank air trials, one at the beginning of the assay and one half way through the assay at the 8^{th} stimulation.

Behavioral analysis

We developed and used custom software (Birgiolas, Jernigan, Gerkin, et al., 2017; B. H. Smith & Burden, 2014) to extract the antennal positions and angles in each video frame. In these experiments we chose to use antennal angle as a proxy for response intensity.

This measure across this study and others shows the most consistent responses to odors using this method (Birgiolas, Jernigan, Gerkin, et al., 2017). However we need to subtract the mean angles when the odor was not present for each video and calculate the difference in antennal angle from this mean for all frames in each stimulus video for each bee. We did this to standardize the response difference from the resting antennal angle, which was different across bees. Next, to then control for the idiosyncrasy of odorant directional responses we took the absolute value of the previous measure for the during-odor stimulation minus either the before- or the after-odor stimulation period to get our final response intensity measures. The angular data was normally distributed and we used an ANOVA with a Tukey HSD post-hoc analysis to compare responses between odors. We used a t-test with a false discovery rate corrected p-value to test if differences between time intervals were statistically different from zero. All analyses and data figures were produced in R (R Core Team 2017) and then combined in Photoshop.

Calcium imaging projection neuron staining protocol

Immobilized honey bees were placed in custom-made plastic harnesses and held in place with wax. We removed a cuticle window dorsal to the joints at the base of the antennal scape and ventral to the medial ocellus in the head capsule of the harnessed bee. We then carefully moved the hypopharyngeal glands and trachea around the vertical lobes to serve as a visual reference for dye injection (Rybak & Menzel, 1993). We then injected one bolus of Fura-2 dextran dye (potassium salt 10,000 MW, ThermoFisher Scientific) coated on the exterior surface of the tip of a glass electrode into each dorsolateral side of the

vertical lobes in the protocerebrum. The injections were intended to damage the lateral antennoprotocerebral tract which contains the axons of uniglomerular projection neurons (Galizia & Rössler, 2010). The dye dissolves in the brain tissue and hemolymph shortly after injection and we then close the head capsule by replacing the window and sealing with eicosane wax. The bees were then fed and dye was allowed to move to the dendrites of the projection neurons into the antennal lobe for approximately 9-14 hours.

Before imaging, we removed the head capsule window once again and removed all hypopharyngeal glands and trachea around the antennal lobes. We rinsed the brain with a Ringer's solution (130 mM NaCl, 6 mM KCl, 4 mM MgCl2, 5mM CaCl2, 160mM sucrose, 25mM glucose, 10mM HEPES, pH 6.7, 500 mOsmol; all chemicals from Sigma-Aldrich). We also cut a small hole between the mandibles and the antennae and pulled out a small portion of a compact structure consisting of muscles, esophagus, and supporting chitin. This procedure helps to prevent small brain movements near the antennal lobes (Mauelshagen, 1993).

Calcium imaging data acquisition

We collected florescence at 340 and 380 nm wavelengths using a CCD camera (SensiCamQE, Till-Photonics) mounted on an upright fluorescence microscope (Olympus BX-50WI, Japan) with a 20x objective NA 0.95 (Olympus), and a 505 nm DRLPXR dichroic mirror and 515 nm LP emission filter (Till-Photonics, Gräfelfing, Germany). We used excitation light alternating between 340 nm for 8 ms and 380 nm for

2 ms emitted from a PolychromeV (Till-Photonics). The camera collected florescence at a rate of 5 Hz over 10 seconds with a resolution of 172 x 130 pxs after a binning of 8x8 on a chip of 1376 x 1040 pxs. This results in a sampling resolution pixel side size of 2.6 μm. Image analysis was done using custom software written in Interactive Data Language (IDL; Research Systems) with routines written by Giovanni Galizia (University of Konstanz, Germany). Each odor stimulus recording consisted of 50 florescence images at both 340 nm and 380 nm excitation wavelengths.

Calcium imaging analysis

We identified a common set of 18 glomeruli within each recording based upon morphology and relative position using reconstructions of the AL and the digital honey bee AL atlas (Flanagan and Mercer 1989, Galizia et al. 1999). We also used a tool provided by Mathias Ditzen (Freie Universitaet Berlin, Germany) to obtain images that represent the degree of correlation between neighboring pixels. The pixels stemming from the same glomerulus should be highly correlated over time and pixels from different glomeruli should be uncorrelated. Using this tool we can obtain an image in which active glomeruli are observed as discrete units separated by contrasting boundaries. We removed recordings from any individuals in which we believed the animal was dying or the staining was unreliable because the signal from the antennal lobe decreased or disappeared in later recordings. This procedure left a final sample size of 11 individuals. We then calculated the relative florescence between the two florescent wavelengths (R_i). Where $R_i = 340_i/380_i * 100$ minus the background florescence ratio ($R_{backround}$)—the

average R_i one sec before odor onset, where $R_{background} = 1/5*(R_{11} + \ldots + R_{15})$, where *i* is the frame number in the florescence images. R_i is proportional to the changes in intracellular calcium concentration (Galizia & Kimmerle, 2004). We term R_i as the calcium response measure. We calculated the mean glomerular calcium response by averaging the calcium response during odor stimulation between the two recording sessions (1 and 2) and averaging those across all glomeruli for all animals. We next measured the glomerular odor pattern consistency by calculating the mean glomerular calcium response for each of the 18 measured glomeruli across the two recording sessions (1 and 2). We then correlated these mean glomerular calcium responses across animals in 7x7 non-repeating matrices (120 unique matrices for octanone, 8 unique matrices for low concentration mixture, and 330 unique matrices for all other odors, as some of the animals did not have reliable measurements for the whole stimulus panel). We calculated the determinants for all of these matrices as a measure for the general level of inter-individual correlation for our samples across all odors. Last, we measured intra-individual glomerular consistency for each odor across glomeruli by correlating the glomerular calcium response for the frames 16-20 during odor stimulation from recording session A and recording session B for all glomeruli in each animal for each odor. For both of these measures we used an ANOVA with Tukey HSD post-hoc analysis to compare the measures across odor stimuli. All analyses and data figures were produced in R (R Core Team, 2017) and then combined in Photoshop (Adobe).

CHAPTER 5

DISCUSSION

In my dissertation research I measure plasticity toward natural odors across three time scales in social bees: short-term memory of a stingless honey bee (chapter 2); long-term influence of odor experience on developmental plasticity (chapter 3); and the current selection pressures by pollinators on the odorant signals flowers produce. (chapter 4) This work uses behavioral responses and both physiological recordings and immunohistochemical staining in the central brain of bees to show that sensory plasticity has impacts on behavior and olfactory development, and imposes selection pressures on olfactory communication in which these animals participate.

MAJOR FINDINGS and IMPLICATIONS

Chapter 2 explored the behavioral plasticity of the defense behaviors of a stingless bee, *Tetragonisca angustula*, on short time scale. From this chapter I describe two main findings: first, short-term experience shapes the response strength of defensive modal action patterns in bees; second, short-term experience can shape both individual- and colony-level behaviors. Chapter 2 laid some of the groundwork for the rest of my dissertation, I found that behavioral responses are clearly modifiable with experience. While responses to pheromones do not completely disappear, I found that the threshold of these responses can be modified through non-associative conditioning. These changes are likely a direct result of changing responses in the central brains of these animals.

In chapter 3 I further explored the idea that olfactory experience shapes the antennal lobe network of bees and that this process shapes the behavior of an animal over longer time scales. As an initial step, I modified the olfactory experience of newly-eclosed adult bees and found that animals with a more similar olfactory foraging experience have a more similar antennal lobe physiology. Bees with reduced olfactory experiences also have what appears to be a less-developed antennal lobe network and also express differences in how they respond on a complex odor mixture variance-learning assay. This finding suggests that a diverse olfactory experience following adult eclosion might be necessary for mature antennal lobe formation. Additionally, these findings support the hypothesis that individual olfactory experience shapes the antennal lobe network of adult honey bees. However, these findings left me with two major questions: (1) are natural mixtures somehow more stimulating to the olfactory nervous system, aiding in final mature development of the antennal lobe; and (2) is there something unique about the way odor mixtures are processed in the antennal lobe?

I address some of these lingering questions in chapter 4, where I investigated select behavioral and physiological responses of honey bees to a natural floral odor and its monomolecular components. Additionally, I tested whether the olfactory nervous system of honey bees has some implicit bias toward complex odor mixtures. Complex odor mixtures are common in floral signaling to bees, but it was unclear if bees are adapted to receive or make use of these complex signals. I found that odor mixtures evoke both a high degree of behavioral response in bees and a highly consistent within-glomerular

response in the antennal lobe. The former serves to attract the attention of bees and the latter would make these odors easier to recognize and recall. These two factors could easily provide a strong selection pressure on flowers to produce mixtures over monomolecular odors to signal to pollinators.

Overall, experience with odors can shape the short-term behavior of individuals and groups of individuals. These odor-driven experiences over long-term periods—on the course of days or weeks through development—will not only shape behavior but also influence how the nervous system of these animals responds to odors in the future. Such experiences may also partially explain sensory biases that individuals exhibit. Some of these biases include a clear behavioral bias toward floral mixtures. Whether the evolution of this bias was driven by floral odors or by sensory preferences in early hymenopteran evolution, this bias will currently pose a selection pressure on flowers to continue to produce mixtures that attract bee pollinators. Additionally, the nervous systems of these animals show more consistent neurophysiological responses to mixtures than to single components over repeated presentations. This would suggest that pollinators should also have an easier time learning and recalling mixtures over single odorants. Together, these last two findings support the hypothesis that there is a strong evolutionary relationship between the complex mixtures signals of flowers (signaling the presence of resources and pollen) and the behavior and physiology of bees receiving the signals. As a whole, this dissertation work shows a strong correlation between an organism's experience and their behavioral and physiological responses to stimuli. Further, it explores how some

functions of an organism's nervous system may show evidence of evolutionary biases within a co-evolutionary relationship.

STRENGTHS, LIMITATIONS, and FUTURE DIRECTIONS

It is very difficult to control the stimuli animals experience in the field, but there are some strong advantages to field experiments. Field experiments allow for a more complete picture of the natural behavioral plasticity of animals in an ecologically relevant context that are lost in laboratory assays. Despite these advantages, few studies focus on the plasticity of social insects in the field, and even less on their behavioral plasticity toward natural odors such as pheromones (Dawson et al., 2016; B Gerber et al., 1996; Gong et al., 2017; Menzel, 1985; Sasaki et al., 2014; Wright et al., 2002; Yunker et al., 1999). By taking field-based approach in Chapter 2, I am both able to better understand how natural colonies behave, and to reinforce the ecological relevance of my study questions regarding behavioral plasticity towards natural odors on short time-scales.

Chapter 2 provided a strong behavioral framework for the rest of my dissertation. I found olfactory experience to natural odor mixtures, even a pheromone, will alter the short-term behavior of bees in the field. However, this is an experimentally "noisy" approach, as I could not control all of the stimuli these colonies experienced when assays were not being performed. Even with this additional variability, I found very clear evidence for behavioral habituation across multiple measures that also likely encompassed multiple behavioral castes.

In chapter 2, I did not mark individuals, and do not know if the same animals were measured at each of the various time points in each colony, nor did I measure the precise size of each colony. However, it is unlikely that all the individuals within the colony had direct experience with each of our short alarm exposures. Thus, I hypothesized that there may be mechanisms that allow for the adaptive modification of colony-level responses to alarm pheromones, though none are currently known. For example, there could be quorum effects on the defense responses of *T. angustula* colonies that could be driven by one or a few experienced individuals, similar to nest site selection in other species of bees and ants (Pratt, 2005; Seeley & Visscher, 2004). Alternatively, there may be mechanisms in the hive that allow for the transfer of alarm response threshold information among guards in the hives of *T. angustula*, similar to the waggle dance for the transfer of location and resource information in honey bees, *Apis mellifera* (von Frisch & Lindauer, 1956).

After establishing that colony behavior can be manipulated in the field with exposure to odors. I next tried to control the experience of a honey bee colony as much as possible. I placed entire colonies into tents and provided all of the external resources they required, associated with an artificial odor, and compared these colonies with freely-foraging ones. This experiment, presented in chapter 3, defined the precise olfactory foraging experiences of all the individuals in tents, but also introduced a number of differences across the freely foraging and tent-restricted hives.

Most notably, the environmental complexity, the visual experience of foraging bees, and the forager flight distance bees traveled were dramatically different between the freely foraging and tent-restricted hives. The tent treatment also likely impacted the forager waggle dance communication profile, as bees generally only perform waggle dances for resources that are at long distances from the hive and that offer highly rewarding resources (Visscher & Seeley, 1982). I did not record any in-hive observations during this treatment; however, there were most likely very few, if any, dances that occurred in the tent hives beyond activation buzzes when fresh resources were placed in the tent (Schneider & Lewis, 2004).

Additionally, there may have been slight differences in the thermal pressures applied to the two hives. There are known effects of larval-rearing temperature on the brain development in honey bees (Groh et al., 2006; Groh, Tautz, & Rössler, 2004), but to my knowledge there are no known effects of temperature on brain development post adult emergence in honey bees. This should be confirmed, however, in follow-up studies. It is also highly unlikely that external hive temperatures had any significant effect on the internal hive temperatures experienced by the bees (Fahrenholz et al., 1989). We did not measure the in-hive temperatures directly, but when honey bees have ready access to both carbohydrates and water resources, they maintain hive temperature across a broad range of external temperatures, including those experience by the bees in Chapter 3 (Cook &

Breed, 2013; Egley & Breed, 2013; Fahrenholz et al., 1989; Jones & Oldroyd, 2006; Kühnholz & Seeley, 1997; Siegel et al., 2005; Starks & Gilley, 1999).

With the current experimental methods in chapter 3, I cannot rule out all of the tent treatment effects on post-eclosion antennal lobe development. The follow-up experiment I would like to perform is an olfactory experience rescue treatment in which tent bees would receive a diverse olfactory experience, both with natural floral odor mixtures and with temporal variation in the odors bees experience across foraging days. This additional experiment would rule out all possible alternative effects of the tent treatment on the antennal lobe development of tent-exposed bees. It would also be worth exploring the effects of this treatment on other brain regions, including the mushroom bodies, lateral protocerebral lobe and the optic lobes, testing for correlates of this deprived experience.

The findings in chapter 3 highlight the potential impacts of natural floral odors on both behavior and olfactory processing in honey bees. This led me to ask if the developmental implications of odor restriction, in addition any potential sensory biases, in bees could provide an evolutionary selective pressure on bee-pollinated angiosperms to produce floral mixtures rather than single chemical odorants. As discussed in chapter 4, natural floral odors generally consist of many chemical components, despite the fact that pollinators easily and generally associate single odorants with floral resources (Knauer & Schiestl, 2015). Thus, I measured the behavioral and neural responses of freely foraging

honey bees to the floral-mixture bouquet of *Brassica rapa* and its individual constituent components in chapter 4.

I chose to use freely foraging bees that had normal olfactory development in chapter 4, however, this also meant I did not know what odors these bees had prior experience with nor what odor preferences these past experience might impose. It is possible some of the constituent components of the *Brassica rapa* floral bouquet might have been present in some of the flowers on which these bees were foraging around the Arizona State University Tempe campus. However, I am confident that the bees did not have exposure to the full *Brassica rapa* odor bouquet, nor did they have experience with any flowers from the *Brassica* genus. First, the *Brassica* genus does not generally grow well in Arizona when not being cultivated and I am not aware of any canola oil production (which comes from *Brassica rapa*) in the state of Arizona. Furthermore, when *Brassica* is being cultivated, the plants are primarily harvested as vegetables and are not allowed to produce flowers. Lastly, bees typically forage at most up to 2 km (Visscher & Seeley, 1982) —and only that far or farther when there are no resources closer—and there are no commercial farms that would grow plants in the *Brassica* genus in any quantity to warrant a quantifiable foraging effort by the bees used in our experiments.

I found strong trends that support the hypothesis that the nervous systems of bees responds more strongly to floral mixtures than to monomolecular chemical odorants. These were trends in which mixtures showed the highest values but were not always

statistically more significant than some of the other *B. rapa* components. Learning experiments, both in free flight and in a restrained setting, would be helpful additional assays that may support these findings. A free-flight preference assay with naïve honey bee foragers using complex artificial mixtures, floral mixtures, and mono-molecular odorants could provide a necessary piece to see if these observed laboratory trends correlate to observations in the field and whether or not this phenomenon is unique to the *B. rapa* floral mixture. Additionally a learning assay testing if mixtures are easier to learn or recall over single odorants will be necessary to see if the final olfactory behavior of the animal correlate with our physiological data.

CONCLUSIONS

This dissertation work builds upon many of the neurophysiological findings in the Smith Lab and other labs. In collaboration with J. Birgiolas (Birgiolas, Jernigan, Gerkin, et al., 2017; Birgiolas, Jernigan, Smith, et al., 2017), I have also created novel tools to measure behavior in insects; these tools have already led to novel findings, some of which are highlighted in chapters 2 and 4. The findings in chapters 3 and 4 leave many open questions and are simply the starting point for a much larger set of future experiments into the olfactory nervous systems of bees.

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