The Dissertation Committee for Rong Ma Certifies that this is the approved version of the following dissertation:

Behavioral and Molecular Mechanisms of Pheromone Transmission in the Honey Bee (*Apis mellifera*)

Committee:

Ulrich Mueller, Supervisor

Johann Hofmann

Lawrence Gilbert

Shalene Jha

Christina Grozinger

Behavioral and Molecular Mechanisms of Pheromone Transmission in the Honey Bee (*Apis mellifera*)

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Behavioral and Molecular Mechanisms of Pheromone Transmission in the Honey Bee (*Apis mellifera*)

Rong Ma, PhD

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Supervisor: Ulrich G. Mueller

The European honey bee (*Apis mellifera*) has a sophisticated system of pheromonal signals that mediates a wide range of behaviors important for their fitness, including reproductive dominance, nest defense, and cooperative brood care. In honey bees, there are two distinct pheromones emitted by larvae, brood pheromone and (E)-beta-ocimene. By integrating behavior, chemical ecology, and transcriptomics, this dissertation analyzes several key stages in signal transmission in a systematic effort to understand how these two pheromones affect behavior, and in the process, generates a synthetic understanding of a highly complex system of communication.

Previous studies have explored behavioral and gene expression patterns related to honey bee pheromones; however, none have compared the roles that two divergent pheromones from a common source play in rapid regulation of foraging behavior. Furthermore, while previous studies have investigated the mechanisms of pheromone detection and the factors involved in regulation of foraging behavior, it remains unclear how individual responses to pheromone exposure scales to colony-level changes in behavior. By investigating the behavioral, physiological, and genomic influences of honey bee chemical communication, this dissertation links phenotypic plasticity in behavior to gene expression profiles in the brain and provides insights into the evolution of a sophisticated chemical language.

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Introduction

Group living offers a variety of benefits over solitary living, including the potential for collective intelligence (Couzin 2009; Berdahl et al. 2013), the reduction of predator risk (Franks et al. 2003; Ward et al. 2011), and the improvement of foraging success (Lihoreau et al. 2016). Animals that forage or travel in groups can exhibit highly coordinated collective behaviors in which groups seemingly move as a single unit, as in shoals of fish, flocks of birds, and swarms of insects (Couzin 2009). There has been a tremendous effort to understand how these collective behaviors emerge from independent decisions of individuals, using computer simulations (Lukeman et al. 2010) and field studies (Attanasi et al. 2014), with a strong focus on the mechanistic basis by which individual members of the group make decisions (Sumpter 2006). Individual based computer simulations modeling such behaviors have revealed that, across species, it is possible for collective behaviors to emerge when individuals follow simple rules, like maintaining distance (Mallon et al. 2001), without the need to identify "informed" individuals or determine relative position in a group (Couzin et al. 2005). Recent studies have begun to add nuance to these simple models, showing how dominance hierarchies improve accuracy of group decisions (Nagy et al. 2010), how quorum threshold sensing reduces error (Franks et al. 2003; Ward et al. 2011), and how decision rules develop over the lifetime of an individual (Hinza & Polavieja 2017). Taken together, there is a growing body of evidence that collective behaviors allow basic information to be integrated across large animal groups and profoundly affects their survival and reproduction.

Collective behavior of animals that move or forage in unison arises from independent decisions of individuals in a group (e.g. Ward et al. 2008). Mechanistic studies of information

transfer have revealed simple local interaction rules explain complex collective behaviors (e.g. midge swarms, fish schools, bird flocks, antelope herds). Studying mechanisms of information transfer is powerful tool that can be applied to understand how animal societies coordinate and function (Charbonneau et al. 2013). While most commonly applied to understand movement of solitary species that aggregate, mechanisms of information transfer can also be used to understand organisms with obligate sociality. Many insects communicate using chemical compounds known as pheromones, which provides a convenient and tractable way to study how mechanisms of information transfer affects social behavior in large insect societies. The European honey bee (*Apis mellifera*) has a complex, nuanced chemical language for mediating diverse behaviors, such as division of labor, brood-rearing, and nest defense (Le Conte & Hefetz 2008; Grozinger 2015). Understanding of coordination of social behavior arises from understanding how a worker acquires information about her colony's needs (Seeley 1986; Seeley 1989).

Honey bees provide a fascinating opportunity to study the ways in which decisions of individuals affect the groups of which they are a part. Honey bee colonies consist of one queen and tens of thousands of daughter-workers, and the collective decision-making of a colony can be thought of as a "swarm intelligence" that emerges from the independent, and sometimes conflicting, decisions of thousands of individual foragers, each with limited information (Seeley 2007). For example, colonies often face the problem of how to allocate foragers in a landscape with spatial and temporal variation in floral resources. Foraging honey bees communicate the distance and direction of profitable floral resource patches using stereotyped pattern of body movements, or "waggle dance," after successful foraging trips (Von Frisch 1965). Through a

combination of adjustments to the waggle dance and interactions with nest mates, foraging recruitment can respond to floral resource quality (Visscher & Seeley 1982; Seeley & Visscher 1988) and various feedback mechanisms (Seeley et al. 2012; Johnson 2010; Tan et al. 2013). Honey bee colonies adjust their selectivity to patch quality during periods of abundance or scarcity, and this colony-level allocation of foragers arises from the independent choices of thousands of foragers (Seeley 1986; Seeley et al. 1991). There are various ways that honey bees have solved the problem of coordinating individuals simultaneously, and one of the most common regulators are volatile pheromones that dissipate through the air to coordinate behavior of many workers, such as alarm pheromones that coordinate defense and larval pheromones that coordinate foraging (Breed et al. 2004; Le Conte & Hefetz 2008). While previous studies have investigated the mechanisms of pheromone detection (Sandoz et al. 2007; Villar et al. 2015) and the factors involved in regulation of foraging behavior (Free 1967; Dreller et al. 1999; Fewell & Winston 1992), it remains unclear how individual responses to pheromone exposure scales to colony-level changes in behavior.

Foraging behavior in honey bees is a dynamic process that responds quickly to spatial and temporal fluctuations in resource availability (Moore et al. 1989), quantity of brood, and quantity of stored pollen (Allen & Jeffree 1956; Free 1967). Honey bees spend the first few weeks of their adult life performing tasks within the hive (e.g., sibling care behaviors) before transitioning to foraging outside the hive, at which point they specialize on pollen or nectar foraging (Winston 1991; Fewell & Page 1993). Foragers collect floral resources (e.g., nectar, pollen) from available plants, then return to the colony to deposit their food loads (Fewell & Winston 1992). The stored pollen is then consumed by bees engaged in parental/sibling care

("nurse" bees) to produce proteinaceous glandular secretions that is then fed to larvae (Crailsheim et al. 1992). Nurse bees feed larvae according to their developmental stage, such that young worker larvae receive royal jelly, a protein rich diet, and older worker larvae receive a mixture of glandular secretions and pollen. Therefore, provision of food resources in the colony is an essential part of cooperative brood care and colony survival.

Because the factors that influence foraging behavior in honeybees are complex (Free 1967), it has been challenging to determine how foragers determine the nutritional demands of brood care (Camazine 1993; Dreller et al. 1999); however, there is a growing body of literature that suggests larval pheromones play an important role (Ma et al. 2016; Pankiw 2004a). Floral resources are important for both overwintering survival and reproduction, so foraging honey bees must balance foraging effort between honey production for overwintering and pollen storage necessary for brood rearing (Free 1967; Fewell & Winston 1992; Fewell & Page Jr. 1993; Allen & Jeffree 1956). Honey bee larvae secrete a variety of compounds that, singly or combined, affect the behavior and physiology of adult workers, which have been grouped into two described larval pheromones—brood ester pheromone and (E)-beta-ocimene (Le Conte & Hefetz 2008; Maisonnasse et al. 2009; Maisonnasse, Lenoir, et al. 2010). These two pheromones differ in their chemical composition and properties, yet each has been shown to elicit brood care behaviors (Sagili & Pankiw 2009; Traynor et al. 2014), suppress worker reproduction (Traynor et al. 2014; Mohammedi et al. 1998), regulate division of labor (Maisonnasse, Lenoir, et al. 2010; Leoncini et al. 2004), and modulate foraging activity (Pankiw et al. 1998; Pankiw 2004a; Ma et al. 2016; Traynor et al. 2017). Due to differences in chemical properties and variation in production over larval development, Maisonnasse et al. (2010) hypothesized that the two larval

pheromones could be used to coordinate behavior of thousands of workers throughout the colony simultaneously, fine-tuning the division of labor in the colony (reviewed in Bortolotti & Costa 2014). These prior observations raise questions about how these pheromonal signals function, whether they differ in the information they convey, and how they affect the behavior of nurses and foragers. To investigate the aforementioned knowledge gap, this dissertation addresses three primary questions. First, what are the effects of volatile EBO on honey bee foraging behavior and colony growth? Second, how are global pheromonal signals transferred in honey bee societies? Finally, how does pheromone exposure change patterns of gene expression in honey bee brains to ultimately regulate task specialization amongst foragers?

Therefore, to answer these questions, this thesis combines behavioral, experimental, neurophysiological, and transcriptomic methods in a systematic effort to understand *how* pheromones regulate behavior, focusing on signal reception, social transmission of signals, and the molecular basis of pheromone communication in the brain.

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Chapter 1: Assessing the Role of β-ocimene in Regulating Foraging Behavior of the Honey Bee, *Apis mellifera*[1]

INTRODUCTION

Many insect species communicate using pheromones, the production of which can elicit both short-term and long-term responses in physiology and behavior in con-specific recipients of pheromone cues. The European honey bee (Apis mellifera) has a particularly complex, nuanced pheromonal language used to mediate diverse physiological and behavioral responses in colony members, including nest defense and cooperative brood care in the worker caste, and reproductive dominance in the queen caste (Le Conte and Hefetz 2008). Pheromones in honey bees often occur as multi-component blends (Pankiw 2004). These blends regularly require all chemical components to be present in specific proportions in order to affect behavior and physiology (Pankiw 2004), although individual pheromone components can sometimes produce separate or partial effects on their own. As such, the behavioral and physiological effects of pheromones on honey bees are considered an emergent property of a pheromone blend, which cannot be produced by its individual components alone (Pankiw 2004). Pheromones can also act as context-dependent signals, varying in their spatial and temporal distribution, synergizing with each other, and targeting multiple receivers in a colony (Slessor et al. 2005; Le Conte and Hefetz 2008; Kocher and Grozinger 2011).

A few pheromone blends have been studied in the context of honey bee foraging behavior. One of the best resolved is brood pheromone (BP), which is composed of 10 esters with low volatility: methyl and ethyl esters of linoleate, linolenate, oleate, palmitate, and stearate (Le Conte et al. 1989, 1990). BP is produced by honey bee larvae at all stages, but the amount produced and the relative proportion of the esters in the blend varies with larval age (Le Conte et al. 1994; Metz

[1] This work has been previously published in the journal *Apidologie* and can be found at https://link.springer.com/article/10.1007/s13592-015-0382-x

et al. 2010). Nurse bees perform a suite of brood care behaviors in response to BP, some of which are attributable to single ester components or subsets of esters from the BP blend (Pankiw 2004). The full 10-component blend of BP has been shown to increase the proportion of pollen foraging to non-pollen foraging workers (Pankiw and Rubink 2002; Sagili et al. 2011). These important physiological effects can only be produced when all BP components are present in specific proportions—a synergistic property of the pheromone. Furthermore, because reproductive division of labor and cooperative brood care are central to honey bees as highly eusocial insects (Bourke 2011), larval pheromones that modulate ovary development and nursing behavior likely play an important role in maintaining sociality in this species.

Despite BP's important role in a honey bee colony, the mechanisms by which BP releases short-term increases in pollen foraging is not well characterized (but see Le Conte et al. 2001; Alaux et al. 2009). BP components are considered non-volatile, so it is assumed that bees need to come in direct contact with BP in order for it to affect worker behavior and physiology (Pankiw 2004; Muenz et al. 2012). Nurse workers constantly make contact with larvae during brood care and feeding behaviors, including feeding larvae with glandular secretions produced from transformed pollen stores (Crailsheim et al. 1992; Pankiw et al. 1998). During brood care activities nurses thus have the opportunity to directly assess the level of BP from multiple larvae, as well as assess the colony-wide availability of stored pollen resources. Although foragers do not regularly come in direct contact with brood, there is evidence that they can also be influenced by exposure to BP. For example, when exposed to synthetic BP, individual foragers show dose-dependent changes in sucrose response thresholds, which are correlated with pollen foraging preferences (Pankiw et al. 1998; Pankiw and Page 2001; Sagili et al. 2011). Similarly, when colonies are exposed to synthetic BP, foragers increase pollen foraging but not nectar foraging activity (Pankiw et al. 1998; Pankiw and Rubink 2002; Metz et al. 2010). Previous studies have also shown that pollen foraging effort is negatively correlated with the amount of stored pollen

resources, but positively correlated with the number of brood present in the hive (Allen and Jeffree 1956; Fewell and Winston 1992; Fewell and Page 1993). Furthermore, in the absence of brood, individual foragers can readily switch from pollen to nectar foraging (Free 1967). Together, these studies show that the level of pollen foraging in a colony depends on the foragers' assessment of the availability and consumption rate of pollen resources. However, the mechanism by which forager honey bees sense the levels of stored pollen or BP in a natural colony setting remains unclear.

Honey bee larvae also emit (E)-β-ocimene (EBO), a volatile monoterpene produced primarily by young larvae in their first through third instar (Maisonnassse et al. 2009, 2010). Like other volatile pheromones, EBO can dissipate quickly. BP and EBO are chemically distinct larval pheromones, but they can induce similar physiological and behavioral changes in nurse bees. For instance, Maisonnasse et al. (2009, 2010) showed that EBO partially suppresses worker ovary development and accelerates the behavioral transition from nursing to foraging, which is similar to the long-term physiological effects of low doses of BP. This transition is delayed in the presence of high doses of BP, however. Despite these similarities, EBO departs from previously investigated brood pheromones in two important ways. First, EBO is highly volatile, which means that it can quickly diffuse throughout the hive, thus removing the need for foragers to come in close physical contact with brood to affect their behavior. Second, EBO is comprised of a single compound, which means that its effects are not dependent on a blend of multiple compounds to elicit a behavioral effect. Maisonnasse et al. (2010) therefore hypothesized that multiple worker castes could potentially assess the concentration of EBO in a colony—possibly as an indication of the number of young brood in the hive—and adjust their behaviors accordingly. Recently, Traynor et al. (2015) showed that synthetic ocimene is able to increase total foraging by approximately 35% and the proportion of pollen foraging by 10% in colonies

without brood. However, the effects of varying ambient EBO levels on hive behavior have yet to be explored in colonies with brood.

In this study, we tested whether non-nursing workers can detect EBO directly and, if so, whether non-nursing workers detect and respond to EBO by modulating their behavior. Specifically, we exposed colonies to a pulse of synthetic EBO and measured the resulting colony-wide foraging activity over the course of four hours. Because EBO can diffuse throughout the hive and is produced by young brood with specific nutritional demands, we hypothesized a priori that treating colonies with EBO would increase overall foraging activity, especially foraging for pollen. We found that exposure to 1 h pulses of EBO moderately increased non-pollen foraging, but not pollen foraging.

METHODS

Honey bee colonies

All experiments and hive manipulations were conducted in July and August 2013 at the Texas A&M University Honey Bee Facility, located at the Riverside Campus, College Station, Texas (30° 6′ N; 96° 32′ W). Two experiments were performed on 1-14 July, and 24 July - 8 August. All honey bee colonies were allowed to forage freely in the environment and had access to the same resources: water from a large nearby pond, nectar and pollen from surrounding farms and natural forage. All bees used in this study were of mixed European races, and their respective queens were naturally mated.

For each of two trials, four honey bee "nucleus" colonies were created from source colonies following standard beekeeping techniques (Winston 1987). Briefly, approximately 5,000 workers and a caged queen were introduced into a plastic 5-frame hive box and the entrance to the hive was sealed. Each hive was provisioned with two frames containing ample honey and pollen resources, two frames of mixed-age brood, and one empty frame to allow the

colonies to expand, if needed. The nucleus boxes were modified in two ways: a wooden landing strip was added to the hive entrance to facilitate the counting of foragers entering the hive, and a petri-dish-sized flap was cut into the side of the box to allow pheromone application with minimal disturbance. All nucleus colonies were placed in an air-conditioned room (~23^{o} C) for 24 h to allow them to assimilate to the new hive environment. The next day, the hives were moved to a new location approximately 100 m away from the source colonies and were placed in a row spaced approximately one meter apart. Tree branches were placed in front of the hive entrances prior to releasing the bees to encourage them to acclimate to the new hive location and to avoid drifting. All queens were released from their cages 2-4 days prior to start of the experiment and were allowed to roam freely around the hive and oviposit.

To estimate the number of bees and food resources present in each hive, we examined all frames individually by taking them out and overlaying a gridded frame consisting of 1 in^2 squares. To assess the initial size of each experimental colony, we counted the number of squares that were covered by workers and by comb containing brood, honey, or pollen prior to the start of each trial. At the beginning of each experiment, the amount of brood, stored food resources, and empty space were equalized in each hive to the initial levels to ensure consistency throughout the course of the experiment. The brood measurements combined the amount of open and capped brood present.

Experimental design

Each day of the experiment, pairs of hives were randomly assigned to either a synthetic β-ocimene pheromone treatment or a paraffin oil control treatment. To minimize colony disturbance, treatments were placed in a petri dish and covered with wire mesh (to avoid direct worker contact

with chemicals) and inserted directly under frames using a small trap door cut into the bottom of the hive box. The pheromone treatment consisted of 1 mL of synthetic β-ocimene (mixture of isomers, Sigma-Aldrich) mixed with paraffin oil for a total volume of 2 mL, while the control treatment was 2 mL of paraffin oil alone.

Treatments were placed in each hive in a pair at the same time for one hour and then removed. The number of foragers returning to the hive was counted for five minutes immediately after the removal of each Petri dish, and then again each hour thereafter for a total of four hours. We recorded the time at which a pheromone treatment was placed in the hive, the time when it was removed, and the time when observations of foragers returning to the hive were made. Thus, it was possible to calculate the elapsed time between foraging observations and the removal of the petri dish containing pheromone or control treatments.

All returning bees were counted as either pollen foragers or non-pollen foragers based on whether they carried pollen in their hind corbiculae. Because foragers were only scored for presence or absence of pollen, foragers that returned without pollen could not be distinguished between nectar and water foragers. Observations were made on sunny days during the same times each day (0900 h to 1700 h). In the first trial (1-14 July, 2013), observations were made only in the afternoon (1300-1700 h). During the second trial (24 July- 8 August 2013), observations were made in the morning and the afternoon to collect more information and to avoid confounding variation in foraging rates due to time of day with response to pheromone treatment. Data were collected on sixteen separate days for a total of 180 observations for each treatment. Observations of returning foragers were made by RM, who was not blinded to a colony's treatment condition during data collection.

Synthetic pheromone and chemical analysis

The synthetic ocimene used in this study (≥90%, Sigma-Aldrich) was a mixture of isomers. (*E*)-β-ocimene was likely present in at least 25% of the total ocimene used (discussed

below). To determine the amount of ocimene that evaporated into the hive, a mixture of ocimene was compared before and after evaporating for the same duration and average temperature inside the colony (35^{o} C) as was done during the experiment. To treat colonies, a dish with mixture of 1 mL of ocimene and 1 mL paraffin oil (2 mL total volume) was inserted into an empty hive box and allowed the mixture to evaporate for 1 h at 35^{o} C. Then the remaining mixture was transferred to a clear glass autosampler vial (Thermo Fisher Scientific, Massachusetts), refilling it to 2mL with paraffin oil and adding $C_{22}H_{46}$ as an internal standard. A 2 mL mixture of paraffin oil and ocimene was treated in the same way, representing a "before-evaporation" sample. Both of these samples were analyzed using gas chromatography - mass spectrometry (GC-MS). The samples were analyzed on a Trace Ultra GC (Thermo Fisher Scientific, Massachusetts), which was equipped with a Restek Rxi (1ms column; 30m length; 0.25mm ID; 0.25 mm film thickness) and connected to a TSQ mass spectrometer (Thermo Fisher Scientific, Massachusetts). The injector temperature was set to 250°C, and the oven was programmed to hold for 1 min at 40°C, then ramp to 300 °C at 15°C/min.

Internal standards were used to control for injection volume when comparing gas chromatogram peaks between samples. The peaks for the internal standard in the samples were nearly identical, so the heights of the gas chromatogram peaks for ocimene were used to determine the difference in ocimene concentration before and after evaporation. This analysis, determined that 10% of the ocimene evaporated into the hive (0.1 mL) using the same exposure duration and temperature found in experimental colonies.

According to previous studies, larvae produce the highest amounts of EBO (18 ng/larva/20 min) in the first three larval stages (Maisonnasse et al. 2010). This means that an

application of 0.1 mL of synthetic (*E*)-β-ocimene under our experimental conditions is approximately equivalent to the amount that 15,000 - 50,000 young honey bee larvae would produce in one day (Maisonnasse et al. 2010). Honey bee queens lay an average of 2,000 eggs per day (Page and Erickson 1988) but have the ability to lay 4,000 or more eggs per day at peak productivity (Page and Metcalf 1984). Thus, a large productive colony may have up to 12,000 larvae in the first three larval instars at any given time. While the amount of synthetic pheromone used in the present study is relatively high, it is consistent with the ratio of larval equivalents of pheromone to number of focal bees used in previous studies to demonstrate the effects of EBO on worker physiology (see Maisonnasse et al. 2009).

Statistical analysis

All statistical analyses were performed in R (v 3.0.2) using the car, lmerTest, and lme4 packages (R Core Team 2014; Fox and Weisberg 2011; Kuznetsova et al. 2013; Bates et al. 2014). All foraging data was analyzed using repeated-measures ANOVA on a generalized linear mixed model with (a) number of pollen, non-pollen, or total foragers as response variables; (b) pheromone treatment and elapsed time since treatment as predictor variables; and (c) hive identity (subject) and date as random effects. Tukey's post-hoc test was used to separate means for significant effects. A generalized linear mixed model was used to incorporate a Poisson distribution for count data. To correct for over-dispersion, a random effect that treated each observation separately (Atkins et al. 2013) was included. Error bars used in the figures represent within-subject standard error of the mean (Morey 2008).

To account for differences between experiments conducted during mornings vs. afternoons, or in early July vs. late July, these factors were included in the model as fixed effects.

Because foraging activity was sampled once per hour for a total of four hours after a treatment, we also included in the model elapsed time since exposure as a variable. If there was no significant difference between morning and afternoon foraging activity on a given trial, morning and afternoon data were pooled based on time elapsed since the end of pheromone treatment rather than the time of day. Similarly, observations from the two trials were pooled if there were no statistical differences between observations between early and late July.

In a separate analysis, we incorporated a binomial distribution to determine whether the ratio of pollen to non-pollen foragers changed in response to pheromone exposure. In this analysis, pollen foragers were treated as "successes" out of the total number of foragers counted per trial, but the same fixed and random effects were used as above.

RESULTS

We used a generalized linear mixed model and ANOVA to analyze total foraging activity and to compare pheromone treatment and control groups. After model reduction, there were statistically significant differences in total foraging activity due to pheromone treatment ($F_{1, 355}$=11.57, P = 7.45 x 10^{-4}), elapsed time since exposure ($F_{4, 352}$=4.97, P = 6.5 x 10^{-4}), and the interaction of trial (i.e., early July vs late July) and pheromone exposure ($F_{2, 354}$=4.97, P = 2.02 x 10^{-3}). There were no significant effects due to morning vs afternoon periods ($F_{2, 352}$ = 2.26, P = 0.06) or due to trial ($F_{2, 352}$ = 0.20, P = 0.94), so data from the two trials were pooled and elapsed time since exposure was used in the model instead of time of day. On average, colonies exposed to EBO treatment had 95.2 (± 3.4) foragers per 5-min observation period, while colonies exposed to control treatment had 88.6 (± 2.3) foragers per 5-min observations.

To better understand the effect of synthetic pheromone on foraging, we then analyzed pollen foraging and non-pollen foraging separately, also using GLMM and ANOVA. Pollen foraging was higher in colonies treated with EBO compared to those treated with a paraffin-only control, but not significantly ($F_{1,355} = 0.69$, $P = 0.41$). However, there was a statistically significant effect of time of day period (Fig. 1.2; ANOVA: $F_{1,355} = 51.8$, $P = 3.69 \times 10^{-12}$; Tukey's post-hoc test: P=0.026) and trial (early vs. late July; $F_{1,355} = 13.1$, $P = 3.44 \times 10^{-4}$), independent of pheromone treatment, so data collected in the morning and afternoon were not pooled (Fig. 1.2).

In contrast, non-pollen foraging was significantly higher in the pheromone treatment than the control treatment (Fig. 1.1; ANOVA: $F_{1, 355}=5.57$, $P = 1.8 \times 10^{-2}$; Tukey's post-hoc test: P=0.016). As in the case of pollen foraging, there was a significant effect of elapsed time since exposure ($F_{2, 352} = 2.38$, $P = 0.05$), although there was no statistically significant interaction between pheromone treatment and time ($F_{4,352} = 0.24$, $P = 0.91$). This indicates that significant differences in total foraging activity due to pheromone treatment may be driven mainly by non-pollen foraging activity.

In a separate analysis, we tested whether the ratio of pollen to non-pollen foragers differed between treatments by incorporating a binomial distribution instead of a Poisson distribution in the GLMM. There was no difference in the ratio of pollen to non-pollen foragers between pheromone-treated hives and untreated controls ($F_{1, 355} = 0.32$, $P = 0.57$), indicating that the ratio of pollen to non-pollen foragers was comparable between the EBO and control treatments.

Interestingly, foragers were more active during some times of the day than others. For example, colonies foraged for more pollen in the early morning than at any other time of day

(Fig. 1.2), irrespective of treatment. Because we allowed bees to forage naturally, it was not possible to distinguish whether patterns of foraging activity over the course of a day were due to our treatments, or were at least in part due to variation in floral resource availability.

The synthetic ocimene that was used in this study was a mixture of three isomers, and it is not clear from the chromatogram which peak corresponded specifically to (*E*)-β-ocimene. There were three isomers present in the chromatogram, so approximately one quarter to one half of the total ocimene present in the sample corresponded to (*E*)-β-ocimene specifically. In addition, it is not clear whether honey bee workers are able detect or distinguish between other ocimene isomers or whether natural larval pheromone contains other isomers.

DISCUSSION

When exposing whole colonies of naturally foraging honey bees to synthetic (*E*)-β-ocimene (EBO) in the presence of natural levels of honey bee brood, exposure to EBO moderately but significantly increases non-pollen foraging in experimental colonies. These observations are consistent with the hypothesis of Maisonnasse et al. (2010) that workers are able to monitor the prevailing EBO concentration in the colony and adjust their foraging behaviors accordingly. Because EBO is a volatile pheromone that dissipates quickly, EBO concentrations in the hive may have a "releaser" effect on foragers, acting as a rapid indicator of the number of young larvae in the colony and, therefore, also of the brood's nutritional demands. We posit that foragers are thus able to sense and respond to the amount of young EBO-emitting larvae currently present in the hive, and that this ability may help scale the colony's foraging effort with the cumulative nutritional demands of the brood.

It is well known that foraging activity in honey bee colonies depends on the amount of brood and stored food resources (Allen and Jeffree 1956, Free 1967, Fewell and Winston 1992;

Fewell and Page 1993). Recently, Traynor et al. (2015) investigated the role that brood of different ages have on foraging activity, including a comparison of colonies with young larvae, broodless colonies exposed to 10,000 larval equivalents of synthetic ocimene, and broodless colonies exposed to a paraffin oil control. Traynor et al. found that, relative to colonies without brood, exposure to ocimene increased both total foraging activity and the proportion of foragers that gathered pollen. The present study, which focuses on the effects of EBO treatment that supplement the brood already present in the colony, found that in the presence of real brood, there is no difference in either pollen foraging or the proportion of pollen foraging when ocimene is added to the colony. Because the absence of real brood has a pronounced effect on foraging behavior (Camazine 1993), our results represent a closer approximation to the effect of EBO in a natural colony odor environment compared to studies using EBO in colonies without brood present. Overall, there is an accumulating body of evidence, including our own data, that the relative proportion of young and old brood plays an important role in regulating foraging activity and that the process is mediated by larval pheromones.

Previous studies on larval pheromones in honey bees have been unsuccessful at determining their mode of transmission from brood to adult workers. Pankiw et al. (1998) assayed foraging behavior in response to brood pheromone (BP) and found support for the idea that foragers could assess BP directly, though the authors could not rule out the possibility that nurse-forager interactions mediated BP-induced foraging behavior. That particular study used hexane extracts of young brood (2^{nd} - 4^{th} larval instars), so EBO could have been a component in the larval extract tested. Later studies using synthetic BP or its individual ester components similarly could not distinguish whether foragers assessed BP directly, or nurse bees transferred

BP to foragers (Pankiw and Page 2001; Metz et al. 2010). In our study, we prevented bees from directly contacting experimental chemicals by applying either the pheromone or control treatments in petri dishes covered with wire mesh. Because workers did not come in direct contact with the EBO in treated colonies, it follows that physical transfer of larval signals to nurses is not required to elicit behavioral changes in foragers. Presently, we cannot determine whether foragers respond to EBO directly, or whether nurses act as a relay to transduce the EBO signal to foragers, however. Regardless of the route, larvae potentially have a line of communication with foragers, and a larval-derived cue can play a modulatory role in determining the rate of non-pollen foraging, but not pollen foraging.

The effects we observed of ocimene on worker foraging behavior were moderate, indicating that, in our experimental paradigm at least, increases in EBO were not sufficiently potent to elicit a drastic increase in foraging rate. There may be two mutually non-exclusive explanations for why we observed moderate effects of EBO on foraging activity. First, the collective foraging rate of workers may be contingent upon additional colony cues (both within and outside the hive), with EBO typically acting in concert with these other cues to determine the foraging rate. For example, EBO may function in combination with, or augment the effects of, brood pheromone (BP), the levels of which were not assayed in our study. Secondly, in our experimental setup, it is possible that the baseline foraging rate in our control and experimental hives was already high for small nucleus hives, so that the effects of treatment with EBO were constrained by the colony's intrinsic high foraging rate. Hence, although we have presented evidence for a modulatory effect of EBO on worker foraging, it remains possible that stronger effects could be observed in hive contexts that differ from our experimental setup. Furthermore,

while we have focused on foraging rate exclusively, how EBO influences the diversity of other honey bee behaviors throughout the hive remains to be tested.

As a volatile pheromone, EBO diffuses quickly throughout the hive, dissipating over time and diminishing in salience. Such an ephemeral cue could act as a releaser that allows foragers to rapidly detect larval presence and nutritional state (i.e., hunger), especially if larvae can modulate the rate of pheromone production or release. Each hive received a 1-h pulse of either EBO or a paraffin oil control, so the concentration of EBO in the hive environment peaked during the initial pheromone pulse and dissipated after its removal. Foragers were therefore exposed to less EBO in the hive after each successive hour of the experiment, and so activity would be predicted to be highest immediately after pheromone treatment. Indeed, pollen foraging showed peak activity directly after pheromone treatment (especially in the afternoon), followed by a gradual decrease back to baseline pre-treatment levels of foraging (Fig. 1.1). We did not observe a gradual decrease in foraging activity for non-pollen foragers, though our time course may have been too short to capture any drop in non-pollen foraging activity. While increasing foraging has clear benefits for colony growth, the ability to quickly down-regulate foraging activity can also be beneficial for colony function. For example, honey bees have been shown to reduce pollen foraging when pollen stores are high (Free 1967; Fewell & Winston 1992) and avoid floral patches when predation risks are high (Tan et al. 2013).

Pheromones are important in understanding the evolution and maintenance of eusociality in honey bees, which is defined by reproductive division of labor, overlapping generations, and cooperative brood care (Le Conte and Hefetz 2008; Bourke 2011). The work that has been done so far on the roles of EBO in honey bee colonies (including our own) suggest that EBO

influences each of these defining characteristics of eusociality. For instance, previous studies have shown that EBO suppresses worker ovary development and accelerates the behavioral transition of workers from nurses to foragers (Maisonnasse et al. 2009, 2010). Furthermore, EBO helps to maintain reproductive division of labor, as it suppresses worker ovary activation, ensuring the presence of overlapping generations by modulating behavioral transitions among castes, and encouraging cooperative brood care by regulating foraging (Maisonnasse et al. 2009, 2010).

As discussed previously, brood pheromone plays many of the same roles as EBO, which begs the question: why are there two distinct sets of pheromones that elicit performance of similar colony functions? Previous authors have suggested that brood pheromone and EBO could be targeted to different subsets of workers in the hive, though this has not been tested. In the context of queen pheromones for example, it has been argued that functional redundancy or reinforcement in pheromone communication is expected to evolve when the cost of reproductive conflict is high (Pankiw 2004; Slessor et al. 2005; Kocher and Grozinger 2011). These potential costs include miscommunication, which exacerbates conflict, or failure of communication, which could result in colony dysfunction. If the production of BP and EBO is considered in the context of functional redundancy or reinforcement of a cue or signals, perhaps EBO production may have played a role in both the maintenance and the evolution of sociality in honey bees by suppressing worker ovary development, which reinforces the reproductive dominance of the queen and reduces within-colony conflict over reproduction.

Future studies should investigate the relationships between BP and EBO, between foraging activity and larval pheromone concentration, and between larval pheromone production

and larval nutritional state. Because young brood are fed royal jelly while older brood are fed a mix pollen and glandular secretions, foragers could use the relative levels of BP and EBO to determine the current nutritional demands of the colony, especially if they are also able to sense availability of stored food resources. EBO releases foraging behaviors, so future studies should also investigate the behaviors of workers performing other important hive functions (e.g., nursing, guarding) throughout the hive as well as its applications in promoting colony health and growth.

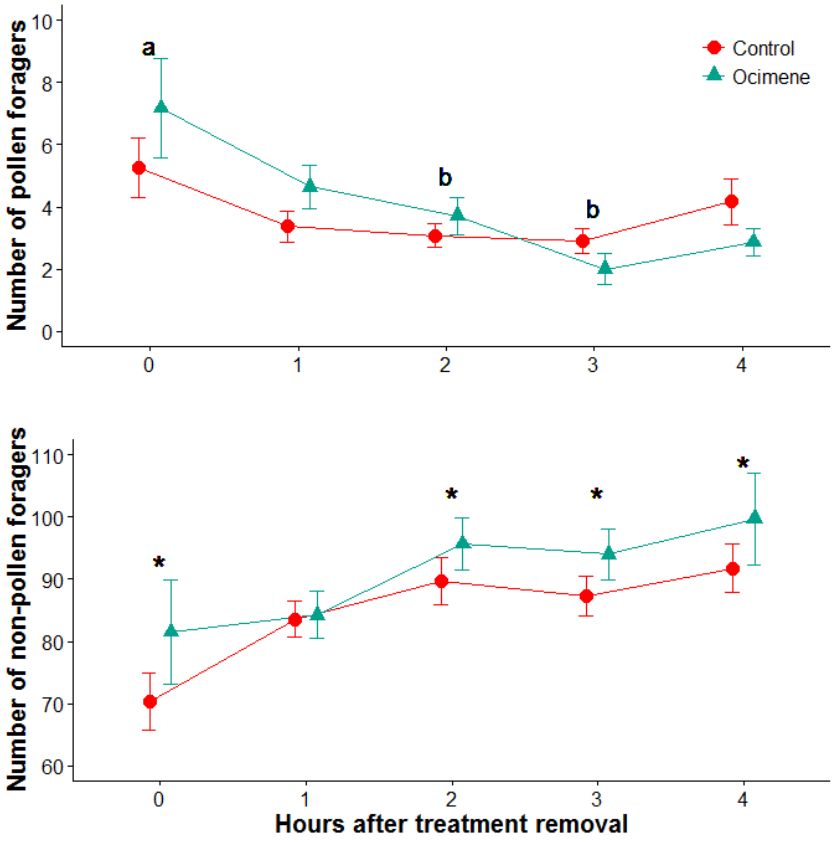

Figure 1.1. **The larval pheromone EBO non-pollen foraging**

Average (± SEM) number of pollen (top) or non-pollen foragers (bottom) returning to honey bee colonies within a 5-min interval after the addition of either an ocimene pheromone treatment or a paraffin oil control. The x-axis represents the number of hours after treatment pulse, either with ocimene (triangle) or a control (circle). Exposure to ocimene significantly increased non-pollen foraging activity compared to paraffin oil controls (ANOVA, $P \leq 0.05$). Pollen foraging showed moderate increases before returning to pre-treatment baseline levels three hours after pheromone treatment. For pollen foraging, different letters represent time points with significantly different foraging activity, independent of foraging activity (post-hoc Tukey's tests: $P= 0.026$ and $P= 0.001$ respectively). For non-pollen foraging, asterisks indicate significant differences in foraging activity based on post-hoc Tukey's tests ($P \leq 0.05$)

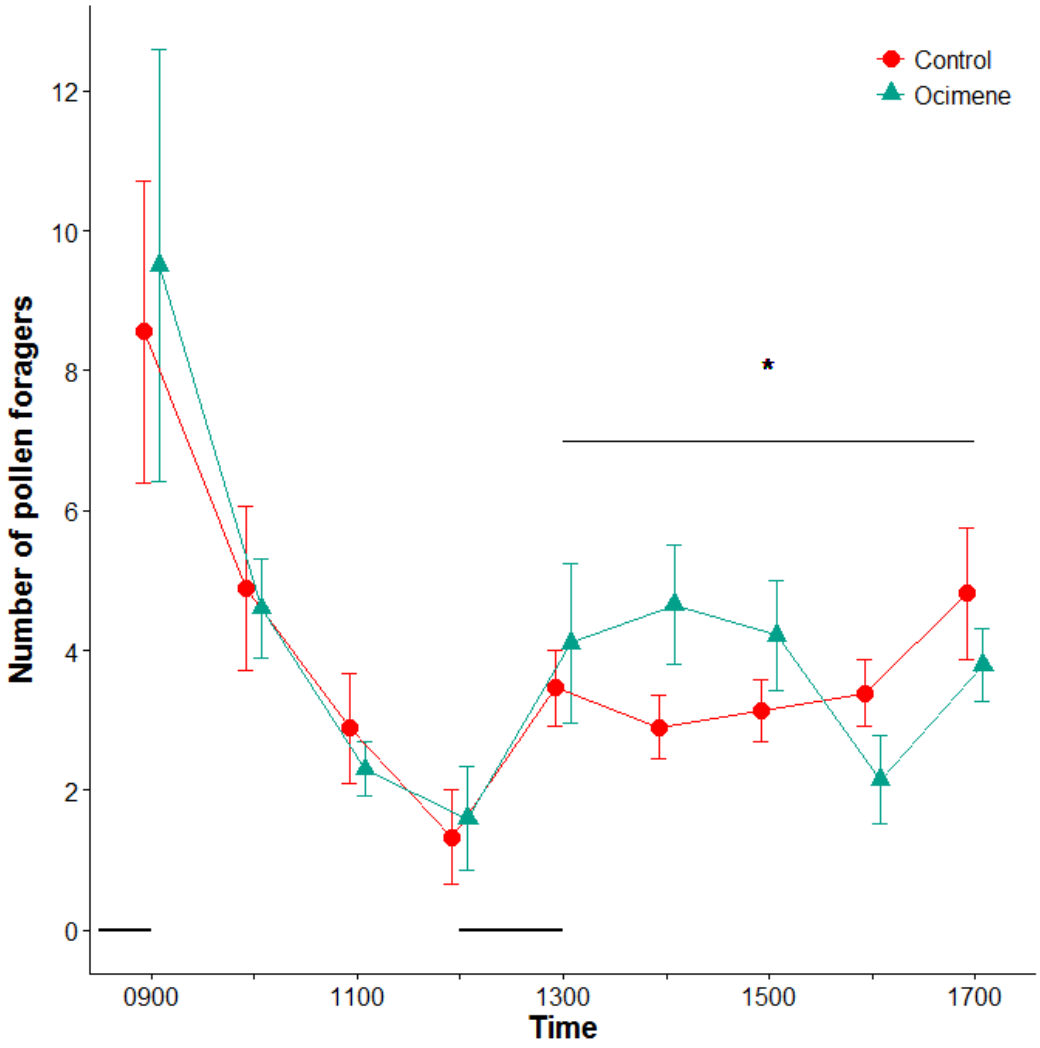

Figure 1.2. **The effect of EBO on foraging activity over time**

Average (± SEM) number of pollen (top) or non-pollen (bottom) foragers returning to colonies within a 5 min-interval after the addition of an ocimene pheromone treatment or a paraffin oil control. Observations began after administering a 1-h pulse of either ocimene or paraffin-only control at 0900 h for morning trials and at 1200 h for afternoon trials. Black horizontal bars represent the time during which EBO was administered. Foraging activity differed significantly between morning and afternoon trials independent of pheromone treatment, and in the afternoon (ANOVA, $P < 0.001$), pheromone-treated colonies showed higher foraging activity than control colonies. Asterisk indicates significant difference in foraging activity between morning and afternoon trials.

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Chapter 2: Behavioral and Neurophysiological Mechanisms of Social Pheromone Transmission

INTRODUCTION

Most studies of collective behavior in animal groups have focused on local interactions between spatially adjacent individuals (Ward et al. 2008). Chemical communication systems allow individuals to rapidly convey information across large, spatially segregated groups (Wyatt 2003; Shorey 2013), including in eusocial insect colonies such as honey bees, where group size can reach 50,000 (Winston 1991). Yet, in most chemical communication systems in social insects, studies have focused on pheromones mediating local interactions amongst individuals performing similar tasks – trail-marking for communication among foragers (Beckers et al. 1989; Czaczkes et al. 2015), alarm pheromone for communication among guards and soldiers (Verheggen et al. 2010), brood pheromones for communication between developing larvae and nurse bees (Conte et al. 1990), or queen pheromone for communication between the queen and her attendants (Van Oystaeyen et al. 2014). Volatile pheromones can mediate interactions between physically distant senders and receivers, and thus could play an important role in global communication networks in social insect colonies. While the function of volatile pheromones in mating interactions in multiple species is well-established (Wyatt 2003; Slessor et al. 2005; Shorey 2013) , their role in mediating social behaviors and organizational structure within social insect colonies is understudied.

A volatile signal could be detected throughout the colony, suggesting that the same pheromone may have colony-wide (global) effects in bees performing diverse tasks. In a decentralized system in which individual bees use local cues to make decisions about how to allocate their time amongst multiple tasks (Seeley 1989), a global signal detectable anywhere in

a colony would have profound effect on task allocation and coordination. While such a global larval pheromone signal has been hypothesized in honey bees (Maisonnasse, Lenoir, et al. 2010), it has not been confirmed that larval pheromones act as direct signals to workers colony-wide; rather, its effects could be mediated through the activities of intermediate bees, such as nurses.

In honey bees, there are two larval pheromones that trigger similar behavioral responses in workers but differ dramatically in their volatility (Table 2.1), providing a tractable system in which to test the role of volatile pheromone transmission in mediating social behavior. The two larval pheromones—brood ester pheromone (BP) and (E)-beta-ocimene (EBO)—share a striking degree of overlap in their effects on adult behavior and physiology both locally and globally. For example, both pheromones have been shown to strong local effects on nurse bees, suppress their reproduction and modulating their behavioral maturation from in-hive to out-of-hive tasks (Winston 1991). Even though foragers do not perform brood care behaviors, both pheromones have a global effect on foragers by upregulating foraging activity (Pankiw 2004a; Pankiw 2004b; Traynor 2014). They BP also promotes a wide variety of local effects on nurse bees, including development of hypopharyngeal glands involved in feeding larvae and initiation of brood care behaviors (e.g., tending, feeding, capping; Conte et al. 1990; Fouks et al. 2011); few local effects have also been shown for EBO (Traynor et al. 2014).

Though they have similar impacts on behavior, BP is considered non-volatile (e.g. Traynor et al. 2017), whereas EBO has high volatility by comparison (Maisonnasse et al. 2009). BP is a blend of 10 different esters with low volatility, while EBO is composed of a single terpene with much higher volatility (Table 2.1). Based on differences in their volatility and their effects on nursing behavior, BP and EBO are thought to differ in mode of transmission through the colony.

Maisonnasse et al (2010) hypothesized that while BP is a "local signal, spread by contact, with a precise action," EBO is "a signal with a global action." However, this hypothesis has not been tested.

Previous studies have suggested that the impacts of queen and brood pheromones on physically distant sets of workers are mediated by local interactions (Naumann et al. 1991; Winston & Slessor 1998). Naumann et al. (1991) used heavy isotope tagged molecules to show that some of the bees attending queen collect her pheromone and disseminate it throughout nest, acting as "messengers" for the queen. The ability of pheromones to be treated as discrete packets of information that rely on worker-worker interactions is useful experimentally because it suggests that if bees can be prevented from participating in these behavioral interaction, the pheromone's effects can be interrupted.

Two studies have investigated the importance of local interactions in transmission of social information related to brood signals, each using wire screens to interrupt access to parts of the hive environment and the bees contained therein (Huang et al. 1989; Dreller et al. 1999). In these studies, a series of wire screens were used to partition the colony into two halves, one with brood and the other without. Nurses could tend the brood, and foragers could leave the colony to forage; however, nurses could not leave the colony, and foragers could not interact with brood. With a single screen, bees on opposite sides of the screen could still interact with, feed, and smell each other. However, with two screens placed in colony, even these limited interactions could be prevented. Importantly, such screens would not prevent the movement of air or the odors it carried. Using this framework, Huang et al. (1989) showed that direct physical interactions are required to affect hypopharyngeal gland development, a long-term change in physiology

associated with brood provisioning; however, they did not test for changes in behavior. In a series of related experiments, Dreller et al. (1999) examined whether local interactions mediated the effects of nurse-forager interactions, stored pollen, number of larvae, and empty space on pollen foraging. However, screen treatments were used inconsistently across individual experiments, confounding their results. After finding no difference between single and double screens in otherwise unmanipulated colonies, double screens were not used in any other treatment. Thus, the effect of nurse-forager interactions could not be excluded in terms of the impact of signals coming from stored pollen, empty space, or brood. In the experiment in which they examined brood signals, Dreller et al. created colonies with low and high brood populations and measured foraging activity over two days, placing the screen in the colony overnight between first and second day. They reported significantly higher pollen foraging in the high larvae treatment colonies on the first day, and no effect of larvae population on the second day after screen insertion, and significantly lower pollen foraging activity on second day overall. This was interpreted to mean that direct interaction between foragers and larvae is required for pollen foraging, since addition of the screen prevented the impact of the larval population size on pollen foraging. However, the significantly lower pollen foraging on the second day indicates that screen treatment and interruption of brood signal were confounded. The result that high and low treatments were not significantly different on the second day could also mean that pollen foragers were trapped in the colony by the screen placement and/or that the study did not have sufficient statistical power to detect a difference. In the end, the importance of local interactions for regulation of foraging remains uncertain.

Here, we use electrophysiology and behavioral assays to compare the transmission modes of two pheromones to test the global signal hypothesis. Because transmission of pheromonal signals depends on the sensitivity of the receiver as much as on the nature of the signal, we measured the ability of individual bees to detect larval pheromones, including bees that are spatially separated in the colony and functionally distinct (i.e. nurses vs foragers). Using established screen methods (Huang et al. 1989; Camazine 1993; Dreller et al. 1999), we then measured the effect of pheromone exposure on colony-level foraging when foragers are allowed full (no screen), partial (one screen), or restricted (two screen) access to frames containing larvae and nurses. Although we predicted that BP would rely on worker-interactions and that EBO would not, our results suggest that both BP and EBO potentially act as global regulators of collective behavior in addition to their local effects on behavior and physiology.

METHODS:

Experiment 1: Neurophysiological response to pheromone exposure

To assess the sensitivity of nurse and forager bees to larval pheromones, we measured average antennal response of nurses and foragers to BP and EBO. We used four pheromone concentrations to represent the natural range of larval pheromone found in honey bee colonies, which spans four orders of magnitude. In total, thirty nurses and thirty foragers were collected from three different source colonies, ten foragers and ten nurses from each colony.

Electroantennography

Both antennae were removed from each bee using small spring scissors and attached to a quadroprobe electroantennogram system (Park et al. 2002; Villar et al. 2015). Clean nitrogen carrier gas was passed over antennae at a constant flow rate. Four dosages of each pheromone were presented in ascending order, and hexane control odor was presented before and after pheromone

odors, and the magnitude of voltage changes were recorded automatically. To account for mechanical stimulation of antennae due to air alone, each response to a pheromone odor stimulation was divided by the average of an individual antennae's response to a control hexane odor stimulation. Therefore, all analyses were performed on relative response to odors, with a value of 1 representing equal response to control as to pheromone odors. All values > 1 provide support that antennae responded more to pheromone odor than to control odor, though there was some neutral variation due to electrical noise of instruments and quality of antennal prep.

Animals and collection

All honey bees used in this experiment were from three separate queenright source colonies at the Chemical Ecology Lab at Penn State University. Nurses were collected while they placed their head and thorax into wax honey comb containing larvae. Foragers were identified as they returned to the colony entrance with pollen loads on their hind legs. Nurses and foragers were collected into separate acrylic cages and kept in an incubator (32°C) until they could be assayed. Smokers, a common beekeeping tool used for calming bees, were not used during beekeeping to prevent bias of smoke on odor preference. Beekeeping was preformed gently to prevent alarm pheromone release. To further ensure that antennae were not exposed to alarm pheromone residues during testing, electroantennography and bee collection were performed by different individuals.

Synthetic pheromones and odor cartridges

Brood pheromone (BP) is a ten-component blend of methyl and ethyl esters of palmitic, oleic, stearic, linoleic, linolenic acids (Table 2.1). While all stages of honey bee larvae and first stages of pupae produce BP, the relative proportions of BP varies with their age (Conte et al. 1990; Metz et al. 2010). The proportions associated with young (1^{st} and 2^{nd} instars) and old (4^{th} and 5^{th} instars) have differing effects on behavior and physiology of worker bees (Metz et al. 2010). Because the blend characteristic of older larvae has been shown to strongly upregulate

pollen foraging(Pankiw 2004a), we used the relative proportional documented for older brood. All components were commercially available (Sigma-Aldrich). Synthetic ocimene was also commercially available (Sigma-Aldrich), albeit as a mix of isomers.

Four biologically relevant dosages of synthetic BP and EBO were used (0.1µg, 1µg, 10 µg, 100 µg), which represent the amount of pheromone produced by 1, 10, 100, and 1000 larvae, respectively, during a single day (Maisonnasse et al. 2009). As such, the dosages used in this study represent 1,10, 100 and 1000 "larval equivalents" of pheromone. Filter paper was impregnated with 10 µL of either analytical grade hexane (Sigma) or a pheromone odor dissolved in hexane. These impregnated strips of filter paper were then carefully placed into clean glass pipettes using forceps. To ensure that pheromone and control odor exposures were standardized, a fixed volume of air was pushed through glass pipettes containing filter paper.

To control for volume of hexane applied to odor cartridges, the concentrations of the solutions were made to 1/10 of the target dosage and 10 uL applied the target pheromone dosage. As such, both pheromones were diluted serially to produce target concentrations of 0.01µg/µL, 0.1µg/µL, 1µg/µL, and 10µg/µL.

Statistics

All statistical analyses were performed in R (R Core Team 2016). Repeated measures ANOVA was used to determine pheromone, dosage, and behavioral state of worker on antennal response. Colony identity and individual identity of workers were included as random variables. The data violated the assumptions of ANOVA, namely homogeneity of variances and normality

of residuals. To confirm ANOVA results, we also used a non-parametric rank-based Scheirer-Ray-Hare test, an analogue of the Kruskal-Wallis test.

Because nurses and foragers could also differ in probability of responding to pheromone concentrations, the data was also analyzed using logistic regression. The data were coded in binary fashion as either response or non-response, such that the ratio of response to pheromone over response to control odor greater than 1 was coded as a response. Thus, logistic regression produces dosage response curves allows evaluation of how likely nurses and foragers are to respond at each pheromone dosage. Logistic regression does not assume homogeneity of variances or normality of residuals, which avoids the issues with ANOVA.

Wilcoxon ranked sum tests (with continuity correction) were used to assess whether bees had greater responses to BP or EBO. Nurses and Foragers were analyzed separately.

Experiment 2: Impacts of pheromone transmission routes on foraging behavior

Experimental Design

Foraging experiments were designed to test whether larval pheromones could directly regulate foraging as a global signal and whether worker-interactions between foragers and nurses were necessary to mediate pheromonal signals (Table 2.3). To assess the importance of brood and worker interactions on collective foraging behavior, foragers were prevented from accessing brood chambers and other bees; then, overall foraging activity for the colony was measured. Single screens physically prevent foragers from access to brood areas, while permitting worker interactions through the screen (e.g., trophallaxis, antennation). Double screen treatments prevent worker interactions in addition to brood areas.

Pheromone treatments

Six colonies were used for each trial. These colonies were randomly assigned to one of three exclusion treatments: screenless control, single screen, double screen. Foraging activity was measured for each colony on three successive days. On the first day of the experiment, each colony was exposed to a control odor (paraffin oil) for one hour, and foraging activity was then recorded to establish a baseline foraging level for each colony. On each successive day, colonies were randomly assigned to either BP or EBO treatment groups, of which they received 5000 larval equivalents through a trapdoor underneath the colony. Larval equivalents refer to the amount of pheromone that a single larva would produce over the course of one day, calculated from previously published emission rates (Maissonasse 2010). Colonies were then given a day of respite in which no pheromone treatments were applied. On the last day, each colony was then exposed to the opposite larval pheromone treatment as they had previously received. In summary, foraging activity was measured for each colony after exposure to control treatment, BP, and EBO. Four replicates of the experiment were performed. In total, twenty-four colonies were used, such that there were 8 colonies in each exclusion treatment (Table 2.3).

Animals and hive construction

For each replicate of trial, six colonies were created from source colonies following standard beekeeping techniques (Winston 1987). The colony entrance was attached to a customized landing strip with a clear acrylic top to allow returning foragers to be filmed when entering the colony. Trap doors were installed under colonies so that pheromone treatments can be placed without disturbing colony, as described previously (Ma et al. 2016). Wire screens were installed in the hive using established protocols to restrict their interactions with nurses and brood (as in Huang et al 1986; Camazine 1993; Dreller 1999). All trials were performed at the honey bee facility at Texas A&M University, Riverside Campus. New colonies were constructed for all trials, and each colony was allocated comparable numbers workers and hive resources.

Each colony was allocated comparable numbers workers and hive resources, as determined by visual inspection through a gridded frame with 1 inch squares. For this experiment, it was important to separate between nurses and foragers. As such, several precautions will be taken to ensure that foraging bees are isolated. Because bees found on brood frames are most likely to be nurses, brood frames (and all bees found on them) will be taken from source colonies and placed immediately behind the screen (if applicable). All other bees in the colony will be placed outside the screened area and allowed to move and forage freely. The area covered by brood, honey, or pollen will be measured for each frame, and special care will be taken to measure capped and open brood since they affect foraging activity. Foraging data was collected on sunny days.

Scoring foraging activity

To record foraging activity, the entrances of each colony was monitored using a series of web cams (Logitech), which allowed simultaneous recording from all colonies. After completion of the experiment, all video recordings were renamed, such that observers who scored foraging activity were blind to screen and pheromone treatments. Foraging activity was scored for five minutes each hour: immediately after initial pheromone exposure, after 1 hour, and after 2 hours. To simplify data collection and subjectivity in determining which bees were foragers, all bees walking directly through the entrance were assumed to be foragers. Foragers were scored for presence or absence of pollen loads, as in previous studies (Ma et al 2016; Traynor et al 2015). Foraging activity was then divided by the number of minutes to produce foraging activity per minute. Because foraging activity varies over time and between days, we used the proportion of pollen foragers to nectar foragers to control for variation in activity across time points.

Statistics

Repeated measures ANOVA was performed to evaluate the influence of exclusion treatments (i.e. social environment), pheromone exposure, and the interaction of exclusion treatments and pheromone exposure in a single analysis. A significant effect of exclusion treatment

in the model would indicate that social environment influences foraging activity, whereas a significant interaction between exclusion and pheromone treatment indicates that the importance of social environment differs between pheromones. Therefore, we included exclusion treatment, pheromone treatments, and interaction effects on foraging activity in the analysis as fixed effects. Colony identity, day, and replicate were included as random variables in the model. ANOVA assumptions were evaluated using Levene's test (homogeneity of variance) and q-q plots (normal distribution of residuals). Post-hoc pairwise comparisons (Tukey's HSD) were performed to better resolve significant results from ANOVA using the lsmeans package, which utilized the same error structure as ANOVA. All statistical analyses were performed in R, RStudio, and associated software packages (car, tidyverse, lme4, Rmisc, lmer, lsmeans).

RESULTS

Neurophysiological responses of nurses and foragers to BP and EBO

To determine whether spatially separated individuals engaged in distinct tasks could detect larval pheromones, we used electroantennography to evaluate the ability of nurses and foragers to detect larval pheromones. After testing 30 nurses and 30 foragers, it was clear that both groups could detect both larval pheromones at a wide range of pheromone concentrations. These concentrations are consistent with the range of larval pheromone found in a natural colony, from the amount that one larvae would produce to the amount that 1000 larvae would produce. Antennal responses increased with dosage (ANOVA, $F_{3,380}=14.8$, $P<0.001$). Though both nurses and foragers responded to the pheromones, across all concentrations of both pheromones, nurses showed significantly greater relative antennal responses (pheromone:control) than forager bees (Fig 2.1A; ANOVA, $F_{1,54}=4.23$, $P=0.045$). Logistic regression reveals that nurses have higher probability of responding than foragers (Fig 2.1B; $z=1.99$, $P=0.047$), indicating a greater proportion of nurse bees responded to both pheromones across the range of biologically relevant

concentrations. Both nurses and foragers showed significantly higher resposnes to EBO than BP (Wilcoxon ranked sum test, W=21021, P=0.007).

The significant interaction between pheromone and dosage indicates that the dosage response curves for the pheromones are different, even though there is no overall difference in response to pheromone alone ($F_{1,379}$=0.2948, P=0.59). Nurse bees have greater responses to both pheromones than foragers. We obtained similar results from non-parametric, rank-based Scheirer-Ray-Hare test, a 2-way analogue to the Kruskal-Wallis test ($H_{pheromone}$=5.667144, df=1, P=0.017; $H_{behavior}$=9.29, df=1, P=0.002).

Impact of pheromone transmission routes on foraging behavior

To test the hypothesis that larval pheromones could act as global signals that influence the behavior without physical contact, we placed increasingly stringent barriers in the colony to prevent foragers from accessing brood chambers and other bees. As expected given previous studies (Pankiw 2004a), both pheromones were effective in increasing the proportion of pollen:non-pollen foraging compared to the same colonies without pheromone treatments on previous days (Table 2.2; Fig. 2.2;ANOVA; $F_{2,208}$=8.7, P=0.0002; Fig 2.1), and pairwise comparisons revealed no difference between pheromones (P=0.33).

Within pheromone treatments, there was no significant effect of screen treatments on proportion pollen foraging (Fig. 2.3; ANOVA; $F_{2,20.9}$=0.0187, P= 0.83), indicating that physical interactions with brood frames or nurses did not affect pollen foraging in response to either pheromone treatment.

DISCUSSION

By studying mechanisms of pheromone transmission at the individual and colony level, we provide evidence that larval pheromones potentially serve as a global signal to foragers in addition to its previously demonstrated role as a local signal to nurses. Based on their differences in volatility and observations that brood pheromone (BP) causes increased behavioral interaction with larvae, we expected that BP acts as a local signal mediated by worker-worker interactions, while (E)-beta-ocimene (EBO) acts as a global signal independent of local interactions. The electrophysiology results demonstrate that although nurses have stronger responses to pheromones at all concentrations, foragers also respond to these pheromones (relatively to controls) across a wide range of biologically relevant concentrations, demonstrating that foragers are still capable of detecting larval pheromonal signals. Moreover, results of the foraging experiment provide evidence that direct interactions of the foragers with the pheromone or with nurses are not required for either larval pheromone to induce pollen foraging. Despite the expectation that BP is a non-volatile pheromone and dependent on physical contact, our results demonstrate that a chemical propriety of a signal cannot by itself be used as a proxy for the magnitude or spatial distribution of behavioral responses (Swaney & Keverne 2009). Taken together, this series of experiments supports the hypothesis that foragers can detect and respond to both larval pheromones without directly interacting with the larvae and without requiring nurses to convey larval signals, suggesting larval pheromones do act as a global regulator of behavior in honey bee society.

Most honey bee interactions studied so far, especially those mediated by pheromone signals, rely on local interactions between bees performing similar tasks, with few examples of truly

global signals. For example, the honey bee queen pheromone informs the colony of the presence of a reproductively active queen. Unless a colony re-queens, the absence of a queen means the eventual death of the colony, so colonies drastically shift their efforts to queen rearing when queen pheromone levels decline after the death of the queen bee. Despite the importance of the queen pheromone to all individuals in a colony, the queen pheromone does not seem to be a global signal. Rather, mechanistic studies have demonstrated that transmission of the queen signal is mediated through direct queen-worker and worker-work contact (Grozinger et al. 2014), including "messenger" bees that physically carry the pheromone throughout the colony, passing it on to other bees and depositing it on the wax as they walk (Seeley 1989). Similarly, larvae elicit a range of behavioral responses from the nurse bees, and foragers recruit each other to floral resources via the waggle dance. Even the alarm pheromone, which is highly volatile and capable of widespread action, primarily causes responses from bees that are already near the entrance or outside the colony. Despite the associated positive feedback that exponentially increases alarm response and stinging behavior, the effects of the alarm pheromone are nevertheless produced by and directed towards a specific segment of the total population: guards and foragers (Pankiw 2004b; Grozinger 2015). In each of these cases, individuals interact with others in closest proximity, and the pheromones that proximate individuals utilize, while generally important to colony survival, is similarly limited in target receivers. Why then, would larval pheromones act as a global signal throughout the colony?

A global signal capable of colony-wide action could prove to be an important regulator of behavior. Honey bees have been adopted as a useful model for understanding social behavior, and their wide range of pheromone signals provides a tractable system for behavioral assays.

Information transfer and decision making in honey bee colonies is decentralized, such that individuals make choices about which tasks to perform based on information passed directly from other bees and indirectly from the hive environment, including airborne odors (Seeley 1989). Our study demonstrates that global signals can be rapid and powerful regulators of behavior, even among individuals too far away from each to use visual or physical signals and cues. Foragers can potentially use the amount of airborne larval pheromone as an indicator for the population size of brood in the hive, and by extension, for growth and reproduction. In response to the same pheromonal signals, nurses feed larvae, tend them, and build wax caps for mature larvae that are ready to pupate. As such, larval pheromones represent a general global signal that triggers valuable – but different – brood-rearing behaviors in two different behavioral states: nurses that feed larvae and foragers that collect food stores used for feeding.

Our study confirms that both EBO and BP release pollen foraging, as previous studies found (Pankiw 2004a; Traynor 2014), but it also raises questions about the function of seemingly redundant pheromones. Despite their similarity, BP and EBO have not previously been directly compared, either in terms of mechanism of information transfer or in strength of effect on foraging. In this series of experiments, we have compared the ability of adults to respond to two larval signals and found few differences between them. Although they seem to have been shown to independently affect aspects of behavior and physiology, perhaps it would be more appropriate to consider them as subsets of a more complex larval signal. As other authors have noted (Maisonnasse, Lenoir, et al. 2010), the combination of BP and EBO would carry information about both the total population of larvae and their age distribution.

While larval pheromones seem to act as a global signal, the downstream behavioral response is dynamic, depending on independent integration of information at individual level. The tasks an individual performs varies characteristically with age and associated physiological changes (i.e. age-based polyethism), transitioning generally from in-hive tasks to more dangerous outdoor tasks (Seeley 1986; Winston 1991). Each member of a honey bee colony is sensitive to a wide variety of information sources, which can be loosely divided into direct sources that involve interactions with nestmates and indirect sources that involve cues from the hive environment (e.g. temperature, humidity, wax characteristics(Seeley 1989; Seeley et al. 1991; Camazine 1993). Individuals must integrate these sources of information with their own physiological state and experience to decide which tasks to perform (Niño et al. 2012), and behavioral observations have revealed that an individual bee performs several tasks in a day in series (Seeley 1989). As such, it is reasonable to expect that changes in animal behavior correspond to changes in integration centers in the brain (Grüter & Keller 2016; Sandoz et al. 2007; Hofmann et al. 2014). Because nurses and foragers often have different responses to pheromones, it is possible that pheromones trigger divergent molecular pathways in their brains, and it is these differences in integration that result in downstream behavioral differences. For example, the larval pheromones BP and EBO have similar effects on foraging but differ in their effects on nurse behavior and ovarian physiology. Therefore, we suggest that larval pheromones activate different sets of molecular pathways in forager brains than nurse brains. Further, BP and EBO may activate the convergent pathways in foragers but divergent pathways in nurses.

The results of our study suggest potential avenues for further investigation. If these pheromones are truly global, as the response of foragers suggest, perhaps bees performing tasks other than nursing or foraging would also respond to larval pheromones. It would be interesting to examine whether bees increase their fanning activity to spread pheromones more effectively through the colony or raise the temperature to promote brood rearing in response to pheromone exposure. Because nurses and foragers seem to differ fundamentally in their response to identical larval signals, there may be molecular pathways in the brain that show context-dependent expression in response to social pheromones. If BP and OC are components of a more complex pheromone, perhaps they would produce synergistic effects on nursing and foraging behavior.

Global signals have a profound potential to regulate the behavior of animal groups and may be more common than previously thought. Studies have focused on local interactions that must be integrated over large groups to affect collective behaviors, which becomes extremely important when large group size precludes interactions with all group members (Conradt & Roper 2005; Sumpter 2006; Ward et al. 2011). Nevertheless, global signals can have truly profound effects when individuals have the ability to communicate widely (Surowiecki 2005; reviewed Conradt & Roper 2005). Honey bees offer tremendous opportunities to study global signals in group sizes that would normally preclude global communication, especially because their chemical signals are experimentally tractable. By investigating the signal transmission of two larval pheromones, we provide insight into transmission of global pheromonal signals. These differences in behavioral responses may be regulated in central processing centers of the brain.

Table 2.1. Vapor pressure of larval pheromone components

Vapor pressure at identical temperature is a measure of volatility. EBO is several orders of magnitude more volatile than BP. Data available publicly through National Institute of standards and technology.

Larval Pheromone	Components	Vapor pressure at 25°C (mm/Hg)
Brood Pheromone (BP)	Methyl Oleate	6.29E-06
	Ethyl Oleate	0.000030
	Methyl Palmitate	0.00006
	Ethyl Palmitate	0.000070
	Methyl Linoleate	0.000009
	Ethyl Linoleate	0.000030
	Methyl Linolenate	*unavailable*
	Ethyl Linolenate	*unavailable*
	Methyl Stearate	1.36E-05
	Ethyl Stearate	0.000030
	Average	**9.95E-06**
(E)-beta-ocimene (EBO)	Beta-ocimene	**1.559**

Table 2.2. ANOVA table for the effect of time and exclusion treatment on pollen foraging

The table shows the proportion of foragers collecting pollen as a response variable, and three factors as predictor variables: Exclusion, Time, Pheromone.

	F	Df	Df.residual	P-value	Significant Difference
(Intercept)	7.7676	1	52.392	0.00739	*
Exclusion	0.1874	2	20.871	0.8305	
Time point	2.7933	2	205.144	0.06355	.
Pheromone	8.7834	2	208.149	0.00022	*

Table 2.3. Design for collective foraging behavior experiment

Colonies were fitted with wire screens to partially limit or fully restrict access that foragers had to nestmates and offspring in brood frames. Foragers could interact with nestmates through a single screen but not through a double screen. No pheromone treatments were administered on first day that foraging activity was recorded. on the second day, each colony was randomly assigned to receive either BP or EBO. On the third day, each colony received the opposite larval pheromone. For example, if a colony received BP on the second day, it received EBO on the third. Diagrams of honey bee and comb represent potential for nestmate interactions and offspring interactions, respectively. Red slash indicates interactions prevented by screen treatments.

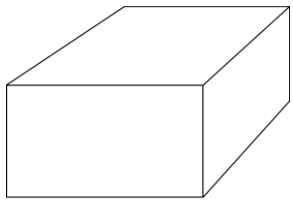
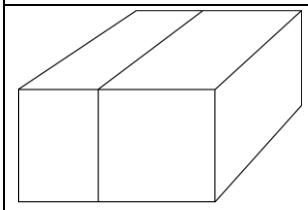
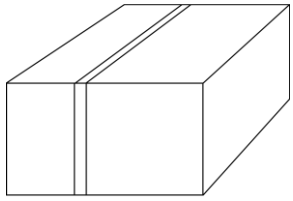
		Pheromone Treatments		
Screen Treatment	**Foragers access:**	**Day 1**	**Day 2**	**Day 3**
 Control	 Nestmates and Brood	Control	Pheromone A: BP or EBO, randomized	Pheromone B
 Single Screen	 Nestmates Only	Control	Pheromone A: BP or EBO, randomized	Pheromone B
 Double Screen	 Neither Nestmates nor Brood	Control	Pheromone A: BP or EBO, randomized	Pheromone B

Table 2.4. Pairwise comparisons between levels of pheromone treatment

The analysis assumes same model and error structure as ANOVA from Table 2.3. BP and OC are both different from control. Asterisks represent significant differences in pairwise comparisons at an alpha level of 0.05.

	estimate	**SE**	**z-ratio**	**P-value**	**Significant Difference**
Control - BP	0.05605	0.018344	-3.056	0.0022	*
Control - EBO	0.07324	0.018283	-4.006	0.0001	*
BP - EBO	0.01719	0.017885	-0.961	0.3365	

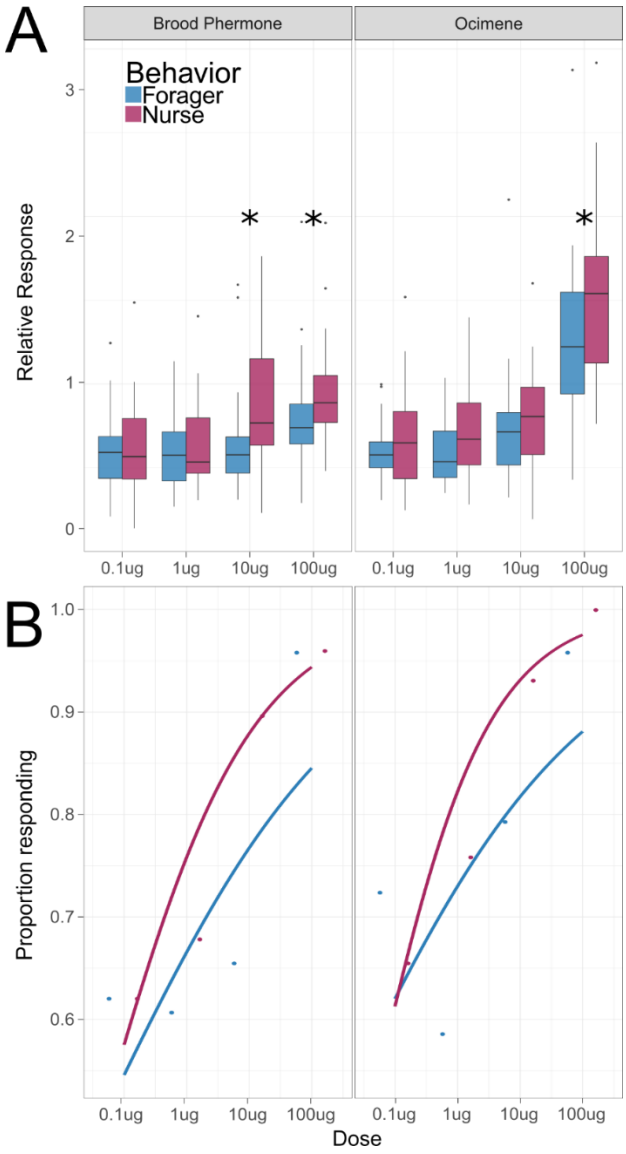


Figure 2.1. **Antennal responses of nurses and foragers to BP and EBO**

Honey bee nurses show greater antennal responses than foragers to both pheromones in terms of magnitude of response (P=0.05: ANOVA) and proportion of individuals that responded (P=0.04; logistic regression). A) Boxplot showing antennal responses to four concentrations of larval pheromone relative to a hexane control. Concentrations represent amounts produced by 1-1000 larvae per day. B) Logistic regression of individuals who responded to larval pheromone at each dosage, showing that pheromones show statically higher across the range.

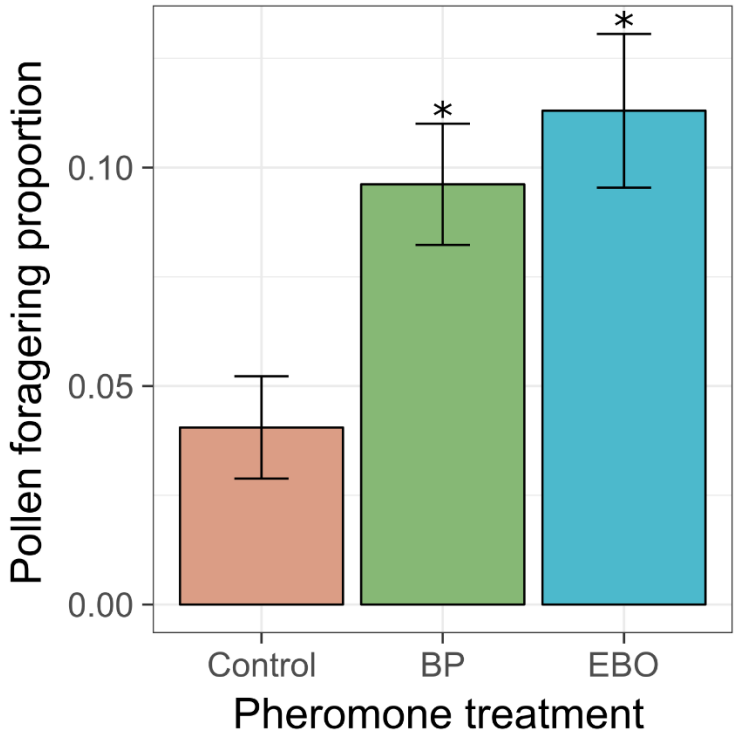


Figure 2.2. **Pheromone treatments increase pollen foraging proportion**

Pheromone treatments increase pollen foraging proportion irrespective of access to brood, verifying that treatments were effective. Asterisks represent significant difference from control ($P<0.05$) based on pairwise comparisons (Table 2.4).

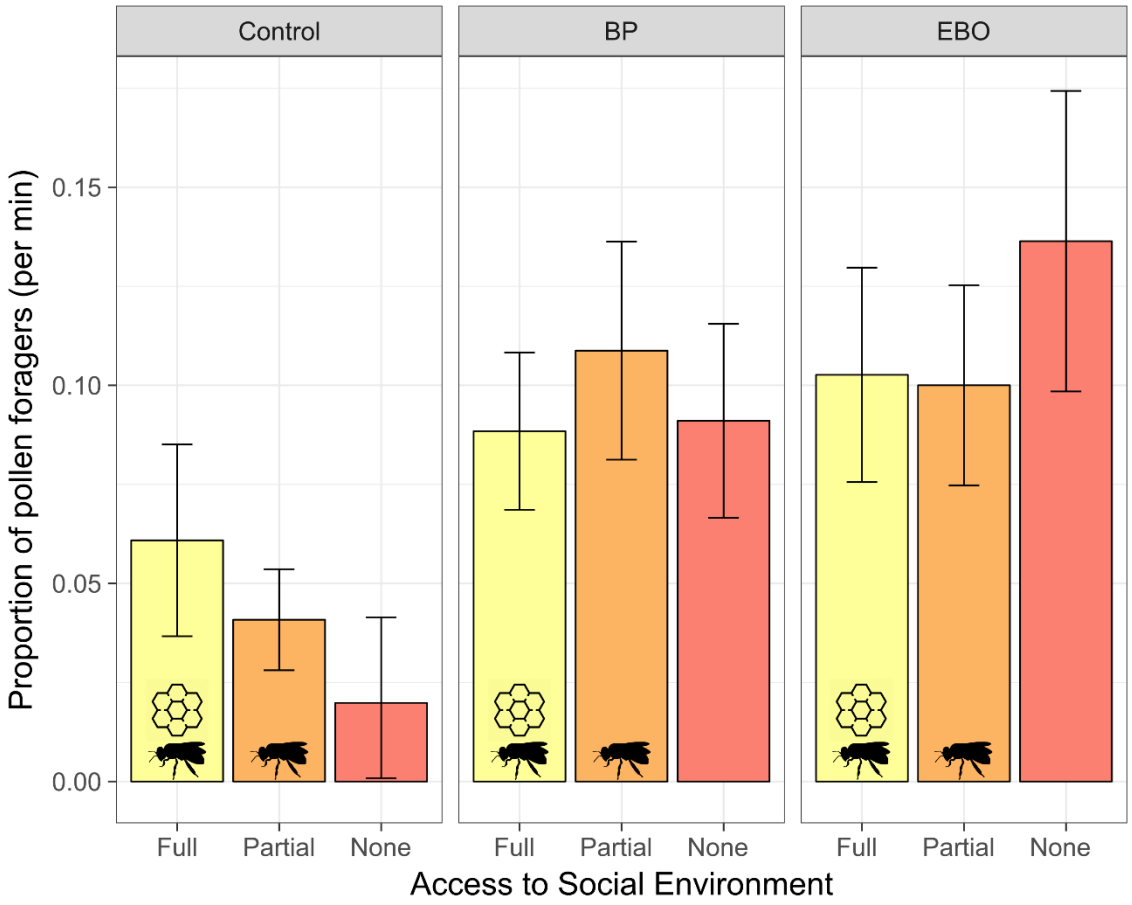


Figure 2.3. **Exclusion treatments do not affect pheromone transmission**

There was no significant effect of screen treatments on proportion pollen foraging (ANOVA; P= 0.83), indicating that physical interactions with brood frames or nurses did not affect pollen foraging for either pheromone. While pheromones were effective in increasing foraging activity, the screens did not affect proportion of foraging within pheromone treatments. The y-axis represents the proportion of pollen foragers, while the x-axis represents the access that foragers have to 1) both nurses and brood (Full), 2) nurses but not brood (Partial), or 3) neither nurses nor brood (None). The panels present data for colonies presented with control, BP, and EBO treatments respectively.

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Ma designed and performed all experiments, analyzed the data, produced figures, and wrote the manuscript. Co-author Villar was also instrumental in designing and performing electrophysiology portion of the chapter.

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Chapter 3: Larval Pheromones Regulate Gene Expression of Task Specialization in Honey Bees (*Apis mellifera*)

INTRODUCTION

One of the hallmarks of insect sociality is a division of labor, where group members specialize on tasks essential to group survival or reproduction (Seeley 2009). Understanding task specialization and division of labor has become a major focus of behavioral research (Simpson 2012; Robinson et al. 2008), and it has become clear that complex animal behaviors are not often regulated by simple genetic or molecular underpinnings, but rather by complex networks of genetic pathways (Ben-Shahar et al. 2002; Ortíz-Barrientos & Noor 2005; Elsik et al. 2014). Further, temporary shifts in behavior or rapid behavioral decisions may be based on differences in response thresholds to signals or environmental cues (Robinson 1987), which may occur at sensory periphery or in integration centers of brain (Joerges et al. 1997). However, many aspects of the molecular basis for rapid behavioral changes remain poorly understood. For example, how does behavioral variation in response to a signal relate to transcriptional patterns in the brain?

There is a growing body of evidence that social behaviors are often founded on conserved neural circuits and molecular pathways across animal species (O'Connell & Hofmann 2012). Because the interactions of social organisms are necessarily mediated through one or more sensory pathways, it has been suggested that the brain is the ultimate regulator of behavior and that neural circuits and integration centers of the brain offer a unique opportunity to study the relationship between genes and behavior (Bloch & Grozinger 2011; Rittschof & Robinson 2014). The olfactory system plays a central role in chemical signaling and communication (Wyatt 2003). Molecules of odor are bound by receptors in olfactory neurons, which may converge in regions of the brain that integrate an animal's physical and social environment with its

physiological state (Grozinger 2015; Symonds & Elgar 2008). In addition to immediate neural responses, transcriptional changes in these integration centers prime the individual, therefore shaping and shifting future behavioral responses (Grozinger et al. 2003; Alaux et al. 2009). These neural circuits and molecular pathways are flexible and dynamic, capable of integrating a wide range of combinations of signals and cues (Joerges et al. 1997). Yet, when two divergent signals produce similar behavioral outcomes, do they necessarily converge on conserved neural and molecular pathways?

Pheromone communication in honey bees provides a convenient system in which to study the molecular basis of task specialization because honey bees produce a variety of chemical signals that mediate a complex behavioral suite, including nest defense, reproductive dominance, and offspring provisioning (Grozinger 2015). Two larvae-produced pheromones, brood pheromone (BP) and (E)-beta-ocimene (EBO), have both been shown to elicit rapid increase in pollen foraging over several hours (Pankiw et al. 1998; Pankiw 2004a); however, their effect on nectar foraging seems to be comparatively weaker (Ma et al. 2016; Traynor 2014). The comparatively stronger effect on pollen foraging emphasizes the importance of pollen nutrients to brood development and maturation of young bees, because pollen is primarily used to feed brood (Haydak 1970). As such, pheromones could possibly serve as a signal that ensures that allocation of foraging effort on pollen versus nectar is commensurate with the colonies investment in overwintering survival versus reproductive fitness.

Previous studies have investigated long-term effects of pheromone on brain gene expression, and on transcriptional changes associated with ontogeny of foraging behavior over the course of individual developmental trajectory; however, these represent long-term changes in physiology

over the course of weeks, not rapid responses to social conditions. In honey bees, pheromones play a key role in the regulation of brain gene expression associated with behavioral transitions over the lifetime of a bee (Grozinger et al. 2003; Alaux & Robinson 2007; Ben-Shahar et al. 2002; C. Alaux et al. 2009; Alaux et al. 2009). Alaux et al. (2009) found approximately 200 genes that were differentially regulated between nurses and foragers after five days of continual BP treatment. These gene expression patterns are associated with long-term change in physiology and behavioral development. While long-term term physiological effects of pheromone exposure are experimentally tractable, gene expression changes have also been demonstrated for rapid behavioral changes in response to brief stimuli (Alaux & Robinson 2007; Alaux et al. 2009). There is some limited experimental precedent for measuring brain gene expression within hours of pheromone exposure; however, the molecular basis of these rapid responses has not been thoroughly investigated, especially in the context of pheromonal regulation.

BP and EBO elicit rapid but divergent changes in behavior among foragers, so this system of larval pheromones provides an opportunity to elucidate the molecular mechanisms underlying variation in decision making and task allocation. Foragers have similar reactions to BP and EBO at the levels of signal reception and transmission (Chapter 2), so perhaps integration of larval pheromone signals in the brain could explain how larval pheromones produce different behavioral shifts in subsets of the foraging population (i.e., via diverging transcriptional pathways). Based on results that BP and EBO have more pronounced effects on pollen foraging than nectar foraging (Traynor 2014), we hypothesize that BP and EBO will induce divergent brain gene expression patterns when comparing foragers specializing on pollen and nectar.

Using two larval pheromones that both increase pollen foraging behavior, we conducted an RNA-seq experiment to investigate the transcriptional pathways in the honey bee brain that are associated with rapid, yet variable behavioral responses. We focus on foragers that specialize on nectar or pollen to test three predications: 1) foragers specializing on pollen and nectar differ in their patterns of gene expression, 2) BP and EBO affect similar transcriptional pathways, and 3) both larval pheromones have more pronounced effects on gene expression in pollen foragers than nectar foragers.

METHODS

Animals and Experimental Design

To avoid differences in behavior and gene expression due to variation in age and genetic background, we created single-cohort colonies from a common source colony with a naturally mated queen; thus, all bees used in the study are half-sisters. Because workers performing any given task in a natural colony can vary widely in age, we constructed single cohort colonies using workers that emerged as adults within a 48-hr period, minimizing differences in age and experience among individuals. After one week, some of the bees in single-cohort colonies transition quickly to foraging (Robinson et al. 1989).

Three such colonies—each provided with identical starting population (1000 bees) and honey and pollen resources—were placed in a large outdoor enclosure (approximately 20’ x 50’ft) at the Texas A & M University Riverside Campus. Each colony was provided with two frames: one frame laden with pollen and honey stores and another frame empty. In addition to frames full of honey and pollen inside the colony, feeders full of sucrose solution and fresh pollen were placed in front of each colony daily, and bees that foraged on each resource were

painted on their thorax. Each colony was also provided a strip of queen pheromone to prevent colonies from developing a "queenless" physiological state and to control for variation in queen quality inherent in using live queens. Colonies did not receive brood. Although broodless colonies are not the default state of a colony, it is nevertheless a natural occurrence when queens die for any number of reasons (Winston 1991). This absence of brood controlled for natural variation in brood signal strength that may have occurred with real brood and minimized the amount of beekeeping interference necessary to maintain identical conditions in the colonies.

After two weeks, colonies were exposed to field-relevant dosages (6000 larval equivalents) of EBO, BP, or a paraffin-oil control. Hive entrances were blocked during the time the pheromone treatment was applied to colony (1 hr), and any foragers outside the colony during that time were removed. When entrances were opened, forager bees (previously marked) were collected as they landed on a feeder but before they initiated feeding. Six pollen foragers and six nectar foragers were collected from each colony and placed immediately in dry ice for later brain dissection. Because two bees were pooled into each RNA sample, sample sizes included a total of 3 pollen forager and 3 nectar forager samples for each of the three colonies (Fig. 3.1; 18 total samples).

Brain Dissection

Individuals were stored at -80°C until dissections, and brains were dissected on dry ice to prevent thawing and degradation of transcripts (as in Grozinger et al. 2003; Alaux et al. 2009), though brains were not freeze-dried. For each sample, RNA from two brains were extracted using RNeasy Mini Kit (Qiagen) per manufacturer protocol and pooled RNA quantity and quality were assayed using Qubit Fluorometer. Library preparation and sequencing were

performed by the Genomic Sequencing and Analysis Facility at the University of Texas at Austin (Illumina HiSeq 4000). All 18 samples were barcoded and split across 4 lanes to control for sequencing bias. A total of 18 RNA-seq single-end 50bp libraries were generated, with 3 libraries for each treatment group from each colony (Fig 3.1).

Data Analysis

Reads were trimmed using Trimmomatic (Bolger et al. 2014) to remove adapter sequences, low-quality reads, and short reads (<36 bp). Sequences were then aligned to an annotated honey bee reference genome (v4.5; Elsik et al. 2014) using default options in TopHat (Elsik et al. 2014; Weinstock et al. 2006; Trapnell et al. 2009). R statistical software and the package DESeq2 (Love et al. 2014) was used to normalize data and make pairwise comparisons amongst treatment groups to identify significantly differentially expressed genes (DEG; FDR<0.05). Gene ontology analysis was performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID v6.8) to better understand biological relevance of differentially expressed transcripts (Huang et al. 2008).

To better understand patterns in the gene expression differences across the sample groups, we selected the genes that were differentially expressed across these pairwise comparisons. The expression patterns for these genes were further analyzed using unsupervised hierarchical clustering and principal component analysis in the base stats package in R. Hierarchical clustering results (Ward method) were visualized using a heatmap with the gplots package (Warnes 2009). Principal component analysis was performed on normalized gene count data to find the linear combinations of genes to explain the maximum amount of variation in the data, producing a series of orthogonal factors. As such, these factors, or principal components,

are uncorrelated and can be considered independently. The first three principle components were visualized using the ggplot2 package (Wickham 2011).

Gene co-expression network analysis

To better understand the groups of genes that contributed to hierarchical clustering results, weighted gene network co-expression analysis (WGCNA) was performed to summarize the variation in gene expression into groups of genes using the WGNCA package in R (Langfelder & Horvath 2008). Differentially expressed genes were normalized in the DEseq2 package and grouped into modules based on similarity of expression patterns (Lutz et al. 2012). Because genes within each module showed very highly correlated patterns, the first principal component of the genes within a module was used to represent the entire module (module 'eigengene'). Then, these module representatives were correlated with sample traits to provide perspective of the function of these co-expressed gene modules (Fig. 3.4). Modules were built with a standardized connectivity score of -2.5. A signed gene co-expression network was constructed with a soft threshold of 10. Modules were not merged. Module eigengenes were correlated with sample traits: pollen vs nectar, BP vs other pheromone treatments, and EBO vs other pheromone treatments.

Overlap of differentially expressed genes with previous studies

Hypergeometric tests were used to assess whether there was a significant overlap of differentially expressed genes with other studies. Specifically, we tested overlap with brood pheromone regulated genes (Alaux et al. 2009) and ontogeny of foraging (Whitfield 2003). These two studies utilized microarrays containing approximately 5500 genes in a previous

version genome assembly. For consistency, microarray probes were mapped to current official honey bee gene set (as in Khamis et al 2015). The degree of overlap between our data and data from these two studies were assessed using hypergeometric tests in the base stats package in R.

RESULTS AND DISCUSSION

Read mapping and differential gene expression

We investigated the gene networks and transcriptional pathways underlying rapid behavioral responses to pheromone signals by comparing gene expression in the brains of pollen and nectar foragers exposed to pheromones. The number of reads per sample varied from 40-90 million (65.8 million average). After quality filtering and adapter trimming, we could map an average of 88% of these reads to the honey bee genome (v4.5;). For additional measures of read quality, see Appendices A and B.

Overall, 2959 honey bee genes had measurable levels of expression and were detected in all samples, so only this subset of genes was used in further analyses. The data were then normalized, and all pairwise comparisons between experimental treatment groups were performed to identify the differentially expressed genes. There were 434 genes that were differentially expressed in at least one pairwise comparison after multiple test correction (FDR<0.05). It should be emphasized that the bees were collected within 2 hours of pheromone exposure, so it is remarkable that our study captured over 400 differentially expressed genes (DEG). To put this in context, Alaux et al. (2009) found ~200 DEG between bees exposed to BP and control bees after 15 days of continuous treatment. The short pheromone exposure duration in our study was necessary to identify genes that are closely associated with behavior rather than long-term changes in physiology.

In our preliminary analyses, it became apparent that each pairwise comparison revealed relatively few DEG, especially when focusing on effect of pheromone treatment while averaging across pollen and nectar foragers, or vice versa. For example, there were 19 genes that were differentially expressed between brood pheromone treatments and control treatments when averaging over both pollen and nectar foragers. However, when considering difference in brood pheromone and control treatments in pollen foragers, there were 30 differentially expressed genes with few overlaps. Our study includes six total sample groups representing all combinations of three pheromone treatments and two forager-type treatments. The statistical consequence of this fact is that we have greater variance between groups of samples and reduced statistical power to detect differences. The number of DEG in our study is, consequently, a conservative estimate of the magnitude of changes caused by pheromone and forager-type. Furthermore, it become apparent that there were strong interactions between pheromone and forager type treatments, and broader analyses than pairwise comparisons would be required to understand the effect of pheromone treatment, forager type, and their interaction.

Foragers specializing on either pollen or nectar differ in gene expression patterns

Studies have investigated the genetic and molecular bases of foraging in honey bees over a range of time scales, yet few have directly addressed transcriptional differences between nectar and pollen foragers, especially in the context of rapid behavioral changes that occur immediately after a stimulus and that last for several hours. There are three pleiotropic quantitative trait loci that are associated with a collection of behaviors collectively known as “pollen hoarding syndrome,” including concentration of nectar collected, and amount of pollen and nectar brought back to the hive (Ruppell et al. 2004). As such, foraging activity has a genetic component;

however, the genetic and transcriptional underpinnings of behavioral plasticity may differ (Ben-Shahar et al. 2002). Young bees spend time within the colony before they transition to foraging tasks (Winston 1991), so many studies have also focused on the ontogeny of foraging over the course of a bee's lifetime (Whitfield 2003; Whitfield et al. 2006; Lutz et al. 2012; Khamis et al. 2015); however, these ontogenetic changes are as much associated with changes in physiology as they are with behavioral plasticity. Finally, Lutz and Robinson (2012) investigated an "immediate early gene" whose expression was upregulated within minutes of initiating an orientation flight, which foragers often perform. Although it has long been established that honey bees specialize on collecting pollen or nectar (Winston 1991), our study is the first to directly compare transcriptional states of pollen and nectar foragers.

Because differential gene expression in our study was produced using several independent pairwise comparisons, we used clustering analysis and principal component analysis to understand broad patterns across all pairwise comparisons. Hierarchical clustering of all differentially expressed genes shows effects of both forager type and pheromone exposure (Figure 3.2). According to the resulting dendrogram (Figure 3.2), the first split among samples separated pollen forager samples and nectar forager samples, regardless of pheromone treatment; however, as is often true with clustering analyses, separation was not perfect. Within these putative foraging branches of the dendrogram, there was a further separation between pheromone and control samples. Furthermore, within each branch of the dendrogram, control samples clearly separated from treated with either pheromone. Interestingly, five out of six brood pheromone samples were associated with the "pollen foraging branch", while a similar proportion of EBO treatment samples were associated with a nectar foraging branch. Therefore,

hierarchical clustering analysis supports the prediction that pollen and nectar foragers differ in gene expression values, but the relationship between pheromone treatments is less clear.

To better understand the contributions of pheromone exposure and forager type on patterns of gene expression, we performed principal component analysis on all differentially expressed genes, revealing the effects of pheromone, forager type, and their interaction (Fig 3.3). Together, the first three principal components (PCs) explain approximately 83.1% of variation in the data. The first principal component corresponds to pheromone treatment and explains 44.9 % of variation, indicating that pheromone exposure is an important factor in individual differences in gene expression. The combination of genes in PC1 were upregulated in response to EBO exposure and downregulated in response to BP exposure independent of forager treatment, while nectar and pollen foragers responded in similar fashion. The second principal component, explaining 22.0 % of the variation, corresponds to forager type, regardless of pheromone treatment. The third principal component explained an additional 16% of variation in the data and represents a pheromone by forager-type interaction.

Results of hierarchical clustering and principal component analysis demonstrate that pollen and nectar foragers indeed show substantial differences in gene expression. We hypothesized that BP and EBO affect different gene expression pathways in pollen foragers than nectar foragers. For this hypothesis to be true, it must first be possible that nectar and pollen foragers regulate different transcriptional pathways. In this sense, the hypothesis is supported by clustering results. There are a multitude of behavioral and environmental differences involved in nectar and pollen foraging tasks. For example, pollen and nectar foragers may visit different floral patches depending on floral resource availability in the environment (Seeley 1986), and

foragers process their food resources differently upon their return to the colony (Haydak 1970; Camazine 1993). Because individuals specialize in pollen or nectar foraging throughout their lifetimes, their gene expression may reflect the cumulative effects of variation in individual experiences (Lutz et al 2012). Therefore, a deeper understanding of the genes and pathways that underlie foraging behavior is necessary to test our hypothesis.

BP and EBO affect similar transcriptional modules

Our second prediction states that the two larval pheromones would regulate similar transcriptional pathways. To elucidate the genes and transcriptional pathways that are regulated by larval pheromones, we utilized weighted gene correlation network analysis (WGNCA). WGNCA can supplement other genomic and bioinformatic methods to provide a more detailed view of molecular processes associated with traits of interest (Langfelder & Horvath 2008; Lutz et al. 2012). WGNCA groups genes into modules based on their patterns of co-expression in multiple individuals (Fig. C.1), independent of sample traits or other elements of experimental details (e.g., pheromone, forager-type). Because modules are formed independently of experimental design, this procedure can identify "significant" modules that show statistically significant correlations with sample traits. Specifically, each module can be summarized by a module "eigengene," which is analogous to the first principal component of genes within the module. The eigengene can then be correlated with pheromone treatment and forager-type to produce correlation coefficient and p-value between each module and sample trait.

We applied WGNCA to the gene expression data and identified 15 highly correlated gene modules (Fig. 3.4). Four of these modules were significantly upregulated in nectar foragers and downregulated in pollen foragers ($P<0.05$), while 2 two modules showed the opposite pattern.

For brood pheromone compared to all other pheromone treatments (EBO + control), two modules were significantly downregulated, and neither was associated with forager-type. Comparing ocimene to all other pheromone types (BP + control), two modules were significantly upregulated, and an additional two were significantly downregulated ($P<0.05$). Interestingly, modules "MEtan" and "MEmagenta" were significantly correlated with both forager type and pheromone exposure (Fig. 3.5). In each of these modules, gene expression showed an interaction effect between pheromone and forager-type: nectar foragers showed greater responses to EBO exposure than pollen foragers and pollen foragers showed greater responses to BP. In other words, EBO seems to make pollen foragers to become more nectar-forager-like, while increasing expression in nectar foragers. BP had the inverse effect, by making nectar foragers more pollen-forager-like. This suggests that one way for pheromones to regulate foraging activity is through a genetic switch between behavioral states. Because bees were collected two hours after pheromone exposure, these transcriptional changes responded rapidly perceived changes in colony and social environment. Such rapid responses in gene expression are plausible given that larval pheromones cause increased foraging activity within an hour of exposure (Pankiw et al. 1998; Chapter 2) and some immediate early genes change their expression within minutes (Lutz & Robinson 2013; Alaux & Robinson 2007). Gene ontology analysis did not reveal any significant terms; however, there was a two-fold enrichment of coiled coil protein domains in "MEtan" ($P=0.045$, Benjamini-corrected). The honey bee foraging gene (*Amfor*) and other similar protein kinases contain coiled coil protein domains (Thamm & Scheiner 2014; Alpadi et al. 2012), as do honey bee silk proteins useful for nest-building (Sutherland et al. 2010; Sehnal & Akai 1990).

Larval pheromones have more pronounced effects on DEG in pollen foragers.

To test the final prediction of our hypothesis that larval pheromones affect gene expression in pollen foragers more strongly than in nectar foragers, we compared overlaps among lists of differentially expressed genes generated in pairwise comparisons. There were consistently twice as many genes in lists from pollen foragers. In all contrasts, there were significant overlaps of genes that were differentially expressed between pheromone treatments when comparing between forager type ($P<0.001$; hypergeometric test; Table 3.1). Despite the increased number of DEG in pollen foragers, BP and EBO regulate overlapping sets genes, which further bolsters the results of gene co-expression analysis. Among the genes that were consistently differentially expressed in response to pheromone treatment were genes related to amino acid metabolism (*pale, henna;* GB48022), homeobox domains, odor conditioning (GB42556), and juvenile hormone binding.

Many of these genes may be related to changes to development, which suggests that even short exposures to pheromone may prime the individual for changes in developmental physiology. Continued exposure to pheromones may cause an accumulation of such developmental genes that serve to bridge the time scale between immediate behaviors and long-term effects that occur after 5 and 15 days of brood pheromone exposure (Alaux et al. 2009). Despite the similarities, larval pheromones do affect a substantial number of DEG that do not overlap within forager types, which is an indication that larval signals have divergent effects in pollen foragers than nectar foragers.

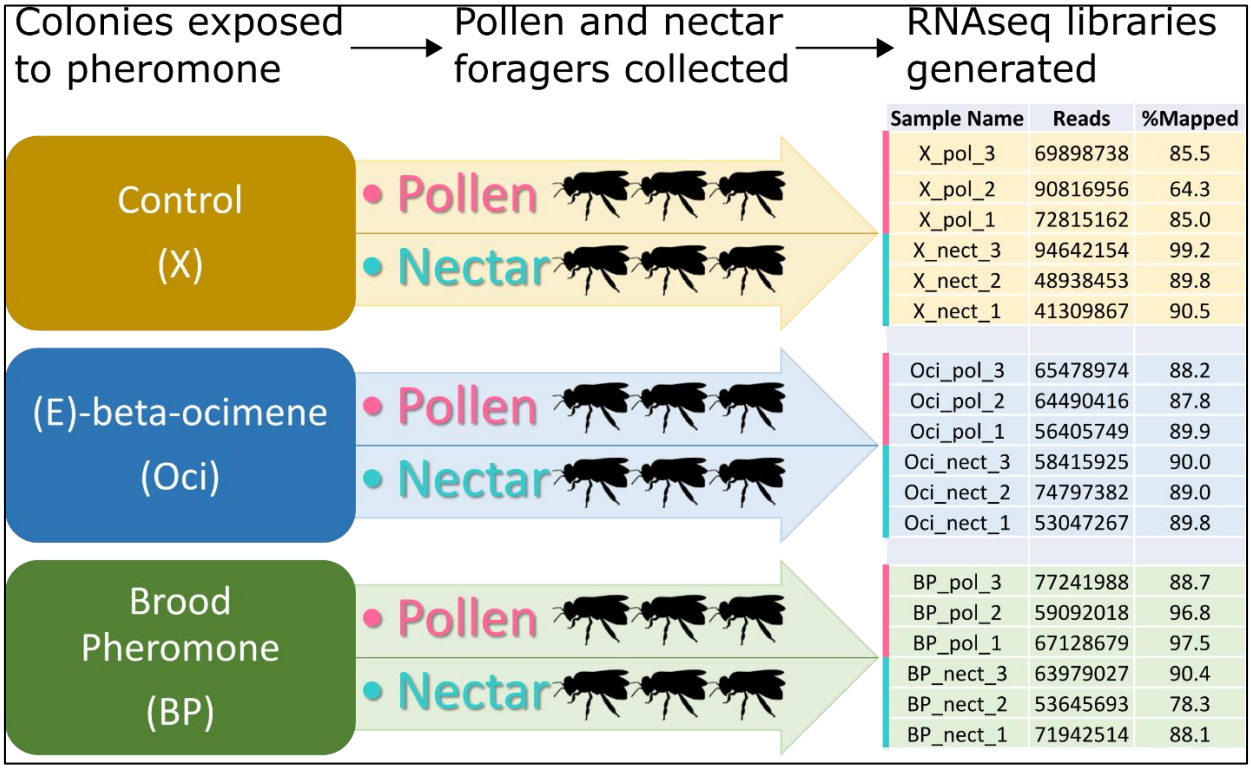
Pheromone treatments also seemed to reduce differences between nectar and pollen foragers. For example, in a pairwise comparison between all nectar forager samples and all

pollen forager samples regardless of pheromone treatment, there were only 18 DEG. While this analysis revealed that chemosensory proteins and glutamate receptors underlie differences between them, the number of DEG seem lows, compared to previous studies that found 1000 genes with expression differences between nurses and foragers (Whitfield 2003). However, pairwise comparisons of pollen and nectar foragers within control pheromone treatments revealed 102 DEGs. As such, pheromone treatments seem to make gene expression profiles more similar. The reduction of differences could be an artifact of averaging gene expression across multiple treatment groups. However, it could also mean that pheromone treatments cause real changes that are opposite in direction, as suggested by WGCNA results (Fig 3.5), which would indicate that changes in social environment have immediate and profound effects on individuals. The gene expression profiles of other social insects have been shown to be similarly sensitive to social stimuli. For example, fire ant foragers become indistinguishable from non-foragers after the queen is removed (Manfredini et al. 2014).

DEG overlap with previous studies.

To explore the relationship between these results and those of previous studies, we compared our data to landmark honey bee transcriptome studies (Whitfield 2003; Alaux et al. 2009). Whitfield et al. compared nurses and foragers, controlling for ontogeny, and found over 1000 DEGs. Alaux et al (2009) was the first to study the effects of brood pheromone on gene expression, and found more than 200 DEGs after 5 and 15 days of pheromone treatments. To test the degree of overlap with previously identified forager genes, we compared the number of DEG between nectar vs pollen foragers in our study, with and without pheromone treatments. When using differences between nectar and pollen foragers across pheromone treatments (18 genes), we found no

significant overlap with the results of Whitfield et al (P=0.23, hypergeometric test). Yet, when we performed the analysis using only the nectar and pollen foragers in the control pheromone treatment, we did find a significant overlap (P=0.043). When testing the degree of overlap with previously identified brood pheromone DEG (Alaux et al. 2009), we used the pairwise comparison between all foragers exposed to brood pheromone and all foragers not exposed to pheromones. In this case, there was a significant overlap (P=0.029, hypergeometric test). The high degree of concordance between our study and the two microarray studies validates not only the expression patterns directly related to foraging and brood pheromone exposure but also those related to task-specific responses to pheromone exposure (i.e. pheromone x forager-type interaction).



Sample Name	Reads	%Mapped
X_pol_3	69898738	85.5
X_pol_2	90816956	64.3
X_pol_1	72815162	85.0
X_nect_3	94642154	99.2
X_nect_2	48938453	89.8
X_nect_1	41309867	90.5
Oci_pol_3	65478974	88.2
Oci_pol_2	64490416	87.8
Oci_pol_1	56405749	89.9
Oci_nect_3	58415925	90.0
Oci_nect_2	74797382	89.0
Oci_nect_1	53047267	89.8
BP_pol_3	77241988	88.7
BP_pol_2	59092018	96.8
BP_pol_1	67128679	97.5
BP_nect_3	63979027	90.4
BP_nect_2	53645693	78.3
BP_nect_1	71942514	88.1

Figure 3.1. **Overview of experimental design and sequencing**

RNA-seq libraries were generated from nectar and pollen foragers exposed to three pheromone treatments. Three pooled pollen forager samples and three pooled nectar forager samples were collected for each pheromone treatment. Each bee diagram represents a sample, though two brains per used for each sample. Resulting numbers of reads per sample and percentages of those reads that mapped to honey bee genome are presented in a table to the right.

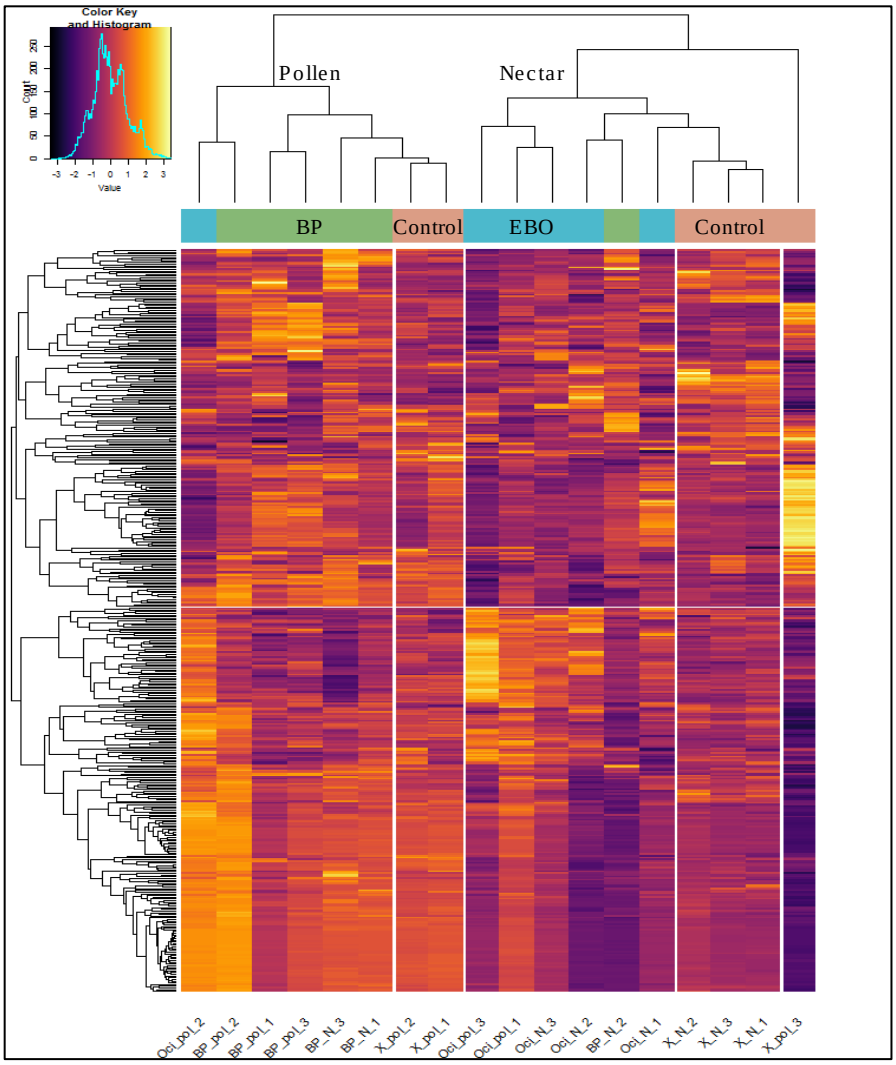


Figure 3.2. **Heatmap for the hierarchical clustering of brain gene profiles**

Honey bees foraging on pollen or nectar were exposed to pheromone treatments: Brood pheromone (BP), Ocimene (Oci or EBO), or Control (X). Rows correspond to 434 differentially expressed genes. Columns represent samples. The scale bar indicates z-scores of gene expression values, with highly expressed genes in lighter colors and low-expressed genes in darker colors. The histogram in the scale bar shows distribution of z-scores. Clustering of samples shows two branches main branches, corresponding to pollen foraging (left) and nectar foraging(right). Within pollen and nectar branches, there is also a split in pheromone treatments.

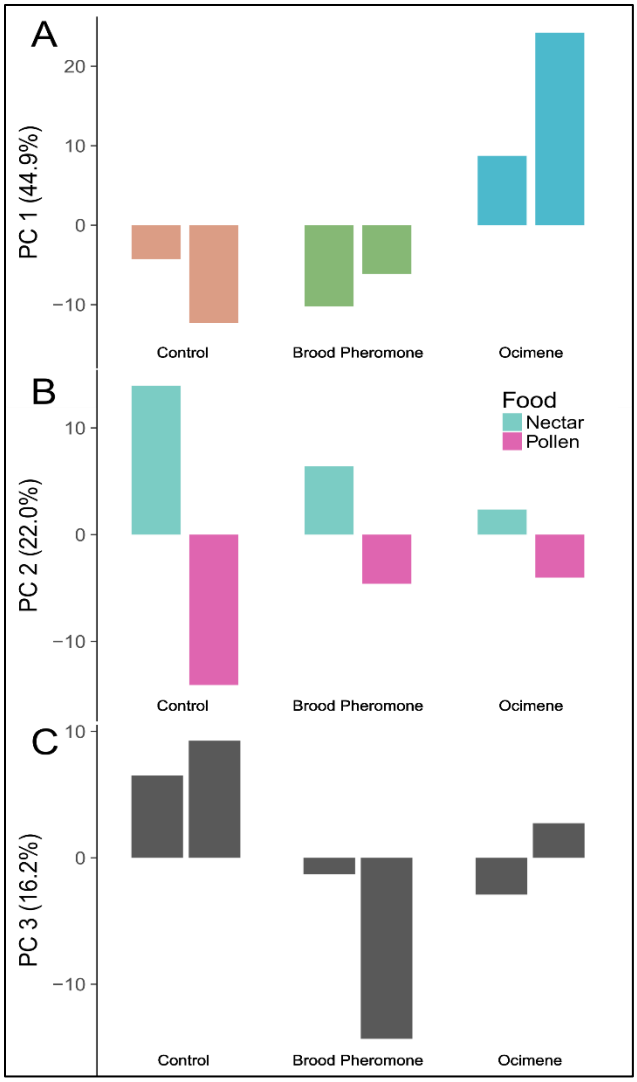


Figure 3.3. **Principal component analysis of all DEG**

Bars represent average expression values for nectar and pollen foragers of each pheromone treatment. The first three principal components (PCs) are displayed, together representing 83.1% of the total variation. A) PC1 corresponds to pheromone treatment. B) PC 2 corresponds to forager specialization (nectar or pollen). C) PC3 corresponds pheromone x forager-type interaction. The percentage of variation in transcript expression patterns explained by each PC is shown in the y-axis.

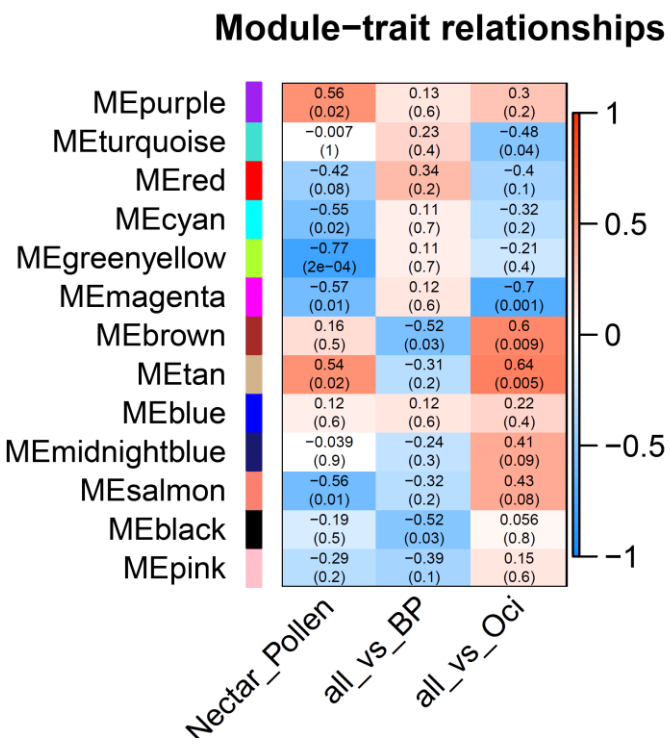


Figure 3.4. **Weighted gene co-expression network analysis**

Rows represent gene modules. Columns represent sample traits. Values in cells are correlations between gene modules and sample types, with associated p-values in parentheses. P-values less than 0.05 represent evidence that there is a biologically meaningful relationship between gene modules and a trait. Red values indicate higher expression in samples associated with pollen, BP, or EBO, respectively. Blue values indicate lower expression in samples associated with those same traits.

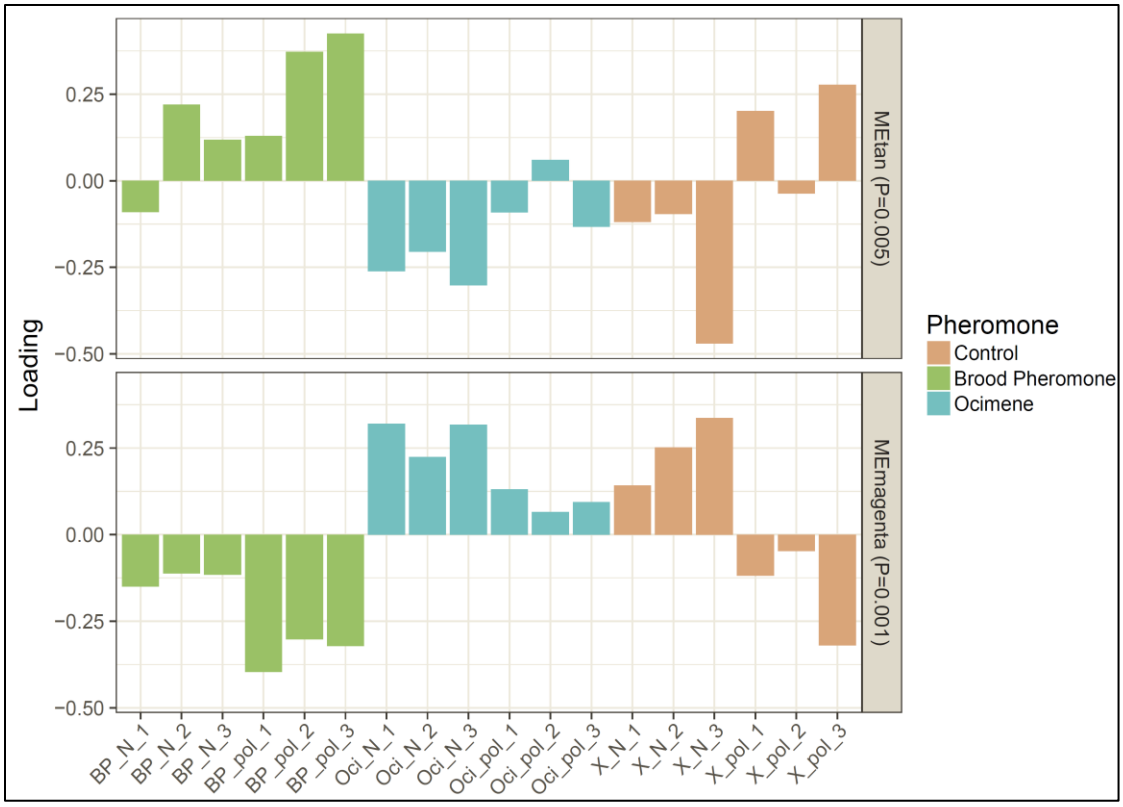


Figure 3.5. **Two gene modules correlated with pheromone and forager type**

Module eigengenes (y-axis), or the first principal component, were plotted for each sample (x-axis). Module name and p-value for correlations with EBO exposure are displayed on the right. Module "MEtan" (top) was downregulated in both ocimene and pollen treatments, while module "MEmagenta" was upregulated in both EBO and pollen. For both modules, pollen foragers in control pheromone treatments showed greater resemblance to BP samples, while nectar foragers showed greater resemblance to EBO.

Table 3.1 Numbers of DEG in comparisons between pheromone vs control treatments, within pollen foraging and within nectar foraging

2959 total genes	Within Nectar Foragers	Within Pollen Foragers	Overlapping Pollen vs Nectar
BP vs Control	22	40	4*
EBO vs Control	46	120	11*
BP vs EBO	28	116	9*

*significantly greater overlap of genes than expected by chance; $P<0.01$; hypergeometric test

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Conclusion

How do animal signals, especially multimodal or complex signals, affect behavior? I addressed this question through a close examination of the mechanisms of communication and resource provisioning in a social animal, the honey bee (*Apis mellifera*). By investigating the behavioral, physiological, and genomic influences of honey bee chemical communication, this dissertation links phenotypic plasticity in behavior to gene expression profiles in the brain and provides insights into the evolution of a sophisticated chemical language.

In pursuit of an answer to the question above, I designed and performed a series of experiments over several field seasons. I found that BP and EBO have similar modes of transmission and effects on foraging behavior, yet the differences in transcriptional regulation I discovered belie this apparent redundancy. Like BP, EBO upregulates foraging activity (Chapter 1) and the proportion of foragers collecting pollen (Chapter 2). Despite their apparent differences in volatility, both pheromones seem to be direct signals between larvae and foragers, even when foragers do not have direct access to larvae or any bee that has interreacted directly with larvae (Chapter 2). In Chapter 3, I found a significant overlap between the genes that are regulated by BP and those regulated by EBO; however, there were groups of genes whose expression patterns showed an interaction between pheromone and forager-type (pollen vs. nectar forager), that is, responses to larval signals also depended on whether the foragers focused on nectar or pollen collection.

Though I approached larval pheromones from primarily proximate levels of analysis, the results have implications for ultimate levels of analysis as well. An understanding of the transmission and integration of honey bee larval signals, a proximate level approach, can help us

to understand the adaptive value and evolutionary history of an elaborate brood signal (NESCent Working Group 2014). In the following sections, I address the implications of the results on our understanding of larval pheromones in terms of their evolutionary history, adaptive value, and complexity.

EVOLUTIONARY HISTORY OF LARVAL PHEROMONES

Many studies have investigated the role of queen pheromones in reproductive division of labor in ants, termites, and bees (Holman et al. 2013; Matsuura et al. 2010; Van Oystaeyen et al. 2014). This likely has much to do with the importance of queen signals in understanding the evolution of eusociality, which is characterized by three main criteria: reproductive division of labor, overlapping generations of breeding individuals, and cooperative brood care. Although cooperative brood care also plays an important role in eusociality, few studies have investigated the role of larval signals in mediating cooperative brood care outside of honey bees (Morel & Meer 1988), and none have done so in a comparative framework. Even though most our understanding of larval pheromones comes from a single species (i.e., the honey bee), comparisons of larval pheromones in the few species for which data are available nevertheless produces some testable hypotheses about the evolutionary history of brood signals.

Pheromone signals in social insects may evolve from systems regulating behaviors that already existed ancestrally (Kocher & Grozinger 2011). For example, queen pheromones may have originated as cues linked to fecundity in a mating context (Kocher & Grozinger 2011). Larval pheromones, on the other hand, may have had their evolutionary origins in cues related to larval survivorship, health, or nutrition. However, it is unclear how adults could assess the health and nutrition of larvae, especially because the ancestral state for brood rearing in bees and wasps

likely prevented larva-worker physical interactions. The life-history and nesting strategies of solitary bees vary widely, but it seems likely that the ancestral state of bee nesting is a form of mass provisioning, in which adults build a cell, provision it with food, seal the cell after oviposition (Roubik 1992). In many species, the adults provision several cells in succession or defend the nest, and in others, adults leave the nest after provisioning cells (Roubik 1992). Adults do not generally interact larvae within sealed cells (Roubik 1992) in either case.

Interestingly, in a mass provisioning sweat bee, *Megalopta genalis* (Halictidae), adult are able to assess the health or survival of larvae even after the brood cell is sealed. Adults bees are able to determine when larvae are sick or dying (Biani et al. 2009; Quiñones & Wcislo 2015), even though they do not have physical contact with larvae and do not perform caretaking behaviors. However, it is not clear whether adults are using odor or vibratory cues to assess larval health or survival. In this light, I hypothesize that the evolutionary origins of larval pheromones are in odors related to infection and health of the larvae. I further hypothesize that larval pheromone evolution and elaboration may have been facilitated by the evolution of progressive provisioning (i.e., workers provisioning larvae continuously through their development), thereby drastically increasing the potential for larva-worker interaction and communication. It has been previously suggested that the transition from mass provisioning to progressive provisioning may promote the evolution of sociality in bees (Schwarz et al. 2003; Field 2005; Field et al. 2004; Queller 1994; Nowak et al. 2010), so examining the relationship between larval signals and life history traits could provide insights into social evolution generally.

Progressive Provisioning Hypothesis

While there is little information available about larval odors of bees, one way to test the hypothesis that larval pheromones co-evolved with progressive provisioning is to survey larval odors in clades with variation in brood provisioning strategy. Honey bees have the best-studied larval pheromones, but there is little information about larval pheromones in other bees in the genus. Although all *Apis* species progressively provision their brood, a survey within this genus would reveal whether larval signals evolved via large saltatory shifts or slow accumulation over time (Symonds & Elgar 2008). After honey bees, bumble bees are perhaps next best-studied system in terms of larval pheromones. Bumble bees, which are closely related to honey bees, seem to rely on cuticular hydrocarbons and do not have a volatile larval signal (Padilla et al. 2016), so it is important to consider whether patterns in *Apis* can be applied broadly to understand signal evolution across bees. Despite the sparseness of our record of larval odors and signals outside of *Apis* and *Bombus*, the allodapine bees may prove to be an especially productive tribe in which to study larval odors because they show variation in both social behavior and brood provisioning strategy (Schwarz et al. 2003; Schwarz et al. 1998; Schwarz et al. 2011). In allodapine bees, adult females exhibit one of three social nesting strategies, depending on the species: 1) lay eggs in the presence of nest mates, 2) join a reproductive hierarchy and delay laying eggs temporarily, 3) support established reproductive individuals by initiating foraging (Schwarz et al. 2011). Among the allodapine bees, there are two lineages that feature mass provisioning, the two sister genera *Halterapis* and *Compsomelissa* (Tierney et al. 2002). It is not known whether allodapine bees use larval cues or signals. In one mass provisioning halictid bee species for which pheromonal information is available (*M. genalis*,

unpublished), there are indications that adults use larval odors to assess health and survivorship of offspring (Biani et al. 2009; Quiñones & Wcislo 2015). In any case, a comparative analysis of larval signals would greatly benefit from the chemical identification of larval odors in additional species.

Floral Diet and Floral Odor Bias Hypotheses

A comparative evolutionary approach can also be used to better understand composition of larval signals. While queen pheromones have been studied intensively in termites, ants, wasps, and bees (Holman et al. 2013; Le Conte & Hefetz 2008; Oi et al. 2015; Van Oystaeyen et al. 2014), larval odors have only been reported in honey bees, halictid bees, and termites, representing three independent evolutions of sociality across millions of years of independent evolution (Conte et al. 1990; Matsuura et al. 2010; unpublished data); yet in all three, there is correspondence between components of queen and larval pheromones (Conte et al. 1990; Matsuura et al. 2010; unpublished data). Additionally, queen and larval pheromones in all three species seem to inhibit or suppress reproduction of nest mates (usually workers; Matsuura et al. 2010; Le Conte & Hefetz 2008). The correspondence of pheromones emitted by brood and by queens is curious, and suggest that queens and brood converged on particular odors, perhaps because these species had an underlying sensitivity to these odors in a different context, or because both queen and larval signals are biosynthetic consequences of a common floral diet (Floral Diet Hypothesis; Table 4.1). For example, the evolution of bees is tied inextricably with the evolution of flowering plants, and bees are sensitive to a wide range of floral odors. In fact, there is a large degree of correspondence between social insect pheromones and floral volatiles. For example, a recent meta-analysis comparing databases of insect pheromone and floral odors

revealed a close relationship between the pheromones that insects produce and the plants they commonly utilize (Schiestl 2010). Because insects that produce overlapping odors sometimes predate the evolution of angiosperms, the overlap was interpreted to mean that flowers evolved to exploit preexisting sensitivity of bees to particular odors, a result that was supported by a subsequent study of plants pollinated by scarab beetles (Schiestl & Dötterl 2012) and by studies of evolution of floral visual displays (Schiestl et al. 2010). This raises fascinating questions about the evolution of chemical communication systems in co-evolved species; however, the data necessary to properly address these questions, such as biosynthetic pathways of pheromones, is largely unavailable in bees. Even so, it is interesting to note that EBO is a monoterpenoid that is common in floral odors (e.g., Piechowski et al. 2010), suggesting the possibility that honey bee larvae evolved the ability to produce pheromones to take advantage of a pre-existing sensitivity to EBO in a foraging context, or conversely, that flowers evolved the ability to produce floral odors that take advantage of a pre-existing sensitivity to EBO in a brood-care context. Because EBO is found in honey bee larvae but not bumble bee larvae, it seems unlikely that the prevalence of EBO in floral odors could be explained by a pre-existing sensitivity in honey bees alone.

The hypothesis that larvae exploited a pre-existing sensitivity in foragers to floral odors therefore seems more plausible. One possible scenario is that queen larvae that produced odor cues reminiscent of floral odors, perhaps as a biosynthetic by-product of pollen-rich diet, received enhanced attention and care from their caretakers, though little is known about the biosynthetic pathways of pheromone production or larval sequestering of floral secondary metabolites. In this scenario, perhaps production of EBO began as by-produced a diet rich in

floral compounds, and the biosynthetic processes involved in digestion were coopted in pheromone production. Unfortunately, data on biosynthesis of compounds like EBO, and the phylogenetic patterns of underlying enzymes that would be necessary to address such evolutionary questions, are not yet available. Our understanding of chemical communication would benefit greatly from identification of social pheromones in a wider set of species, especially in bee clades featuring variation in nesting, provisioning, and life-history strategies.

One Signal Hypothesis

BP and EBO have so far been considered separate pheromones both in this dissertation and in the literature generally. After considering the subtle differences between their effects on behavior and physiology, we must ask, are these subtle differences enough to justify their continued treatment as independent pheromones? BP was identified about two decades earlier than EBO, and it seems their independent consideration is primarily historical. When BP was discovered in the 1990s, ten components were described from hexane extracts of live larvae. As a compound with much higher volatility, EBO would likely not have left much residue on the surface of the cuticle and may not have shown up on GCMS analyses above background noise. Since the identification of EBO (Maisonnasse et al. 2009), the focus has been on its effects on behavior and physiology. As such, it has not been critically evaluated whether EBO and BP truly operate independently or whether they are both components of the same signal.

Although BP and EBO differ in volatility and effects on transcriptional regulation, they can be considered parts of the same multicomponent, multifunctional larval signal, whose effects depend on the physiology of the receiver. Honey bee pheromones are often complex, multi-component blends whose individual components can be produced in different glands and have

effects that are independent of the full blend (Slessor et al. 2005; Pankiw 2004b). For example, the first pass at identification of queen pheromone described five compounds produced in the queen's mandibular glands, called the Queen mandibular pheromone (QMP). These five components elicit retinue behavior from workers, in which they lick and antennate the queen, and suppress ovary development of workers (Slessor et al. 1988); however, they are not as effective as a live queen (Slessor et al. 1988). In subsequent studies, an additional four components from other parts of the queens body were identified (Keeling et al. 2003). When these new components were added to QMP, the resulting blend, called the queen retinue pheromone (QRP); yet, even the QRP is not as effective as a live queen, and there are likely to be additional components (Keeling et al. 2003). Just as QMP is a subset of QRP, perhaps BP and EBO are subsets of a more complex larval pheromone, regardless of their differences in chemical properties, glandular origins, or behavioral effects. Furthermore, BP has already been shown to vary in the relative proportions of its components, so the fact that EBO has an earlier peak-production timing than other BP components is not without precedent. Therefore, I hypothesize that BP and EBO are subsets of a more complex brood signal, which would predict that BP and EBO would have additive or synergistic effects on behavior and physiology of workers. Because it has already been established that BP and EBO affect ovary activation, ontogenetic transition from within-hive to out-of-hive roles, and foraging behavior, these would be the most obvious places to look for synergies between BP and EBO.

Just as fluctuating relative proportions of BP are indicative of larval age (e.g., young vs old profiles), changes in relative proportions of EBO to other BP components could indicate demography of developing brood. The production of larval pheromone components is tightly

correlated with physiological processes in developing larvae (Traynor 2014), and subsets of the total larval pheromone mediate caretaking behaviors at key stages in larval development, such as capping (Le Conte et al. 1995). Thus, a multi-component larval signals that indicates both larval age and demography may permit honey bee colonies to better modulate growth of the colony.

Extended Phenotype Hypothesis

If BP and EBO are indeed subsets of a common brood signal, why did this signal evolve to be so complex (i.e., why almost a dozen components, why not only a few components)? As signals, pheromones are subject to the forces of natural and sexual selection, so pheromone complexity can arise for a variety of reasons, such as mediation of cooperation and conflict. Given the long list of behavioral and physiological effects of BP and EBO, it is reasonable to suspect that larval signals may provide some level of adaptive value in the mediation of cooperation or conflict in honey bee societies. In fact, larval pheromones would not be the first or only socially important pheromone in honey bee societies. The queen pheromone enables queens to maintain reproductive dominance in the colony, and a large body of evolutionary theory has developed around whether queen pheromones is actually honest or dishonest (Kocher & Grozinger 2011; Heinze & D'Ettorre 2009). It is not clear that these queen pheromone hypotheses apply to larval signals, because it is in a larva's best interest to elicit brood care behaviors in both cases. Yet, workers could inspect brood or brood signals as a way to ascertain queen reproductive traits, such as the fecundity or mating number (Kocher & Grozinger 2011). From this perspective, perhaps brood signals could be considered an extended queen phenotype (Extended Phenotype Hypothesis; Table 4.1).

ADAPTIVE VALUE OF LARVAL PHEROMONES

Infection Status Hypothesis

To test the hypothesis that larval pheromones evolved from cues related to health or infection, a comparative approach can be used to evaluate whether there sick and healthy larvae produce different odors and whether there are similarities in these odors across species. After surveys of the natural odors of healthy larvae across developmental stages, larvae of each species can be injured or allowed to develop infection through inoculation and surveyed again. Odors identified in this way could be analyzed 1) in a comparative framework to determine whether there are odors that indicate health or infection across species 2) using bioassays to determine whether adults are attuned to odors related to larval health and survival.

Foraging Tradeoff Hypothesis

From an adaptive value perspective, perhaps one explanation for the existence of a complex, multi-component larval pheromone is that nectar and pollen foragers respond differently to components of the blend (e.g., BP vs. EBO), but why would nectar and pollen foragers respond differently? Perhaps one answer to this question lies in the tradeoff between reproduction and survival. Honey bee colonies face a tough problem every year, in which colonies must collect 1) pollen to quickly produce the thousands of workers necessary to collect ephemeral floral resources and 2) nectar to produce enough honey stores to enable survival in times of dearth (e.g., harsh winters in temperate climates or dry seasons in tropical climates). The difference in gene expression between nectar and pollen foragers could indicate a link between pheromone exposure and relative investment into reproduction and survival. Pollen foragers and nectar foragers both receive the same brood signals, so their differing transcriptional responses to

pheromone exposure could indicate that brood signals have different implications for these two tasks.

A strong brood signal, emitted collectively by thousands of larvae, predicts a growing future work force, which may necessitate increased honey reserves to survive the winter. Nectar foragers may need to undergo physiological changes that enable increased honey collection, such as larger flight muscles for longer and more frequent foraging flights or lower threshold for nectar sweetness. On the other hand, a strong brood signal also means that there is an immediate need for large amounts of pollen to feed thousands of larvae, so pollen foragers may need to increase foraging effort, and among other things, that nectar foragers may need to transition to pollen foragers through changes in gene-expression and physiology. In either case, changes in transcriptional regulation linking short-term behavioral changes and long term physiological change have begun even two-hours after pheromone exposure.

Worker Policing Hypothesis

An alternative hypothesis about the adaptive value of larval pheromones is that reproductive physiology is linked to egg cannibalism, and by producing signals that suppress worker ovary development, larvae increase their survivorship. Reproductive physiology has been linked to egg cannibalism in at least two scenarios: worker policing and anarchistic bees (Beekman & Oldroyd 2008; Beekman & Ratnieks 2003; Ayasse & Paxton 2008). First, workers in queenright colonies police the egg-laying of other workers by eating worker-laid eggs (Ayasse & Paxton 2008). Second, anarchistic worker bees develop their ovaries even in the presence of a queen, and mark their eggs to avoid worker policing and cannibalism (Beekman & Oldroyd 2008; Beekman & Ratnieks 2003). In both these scenarios, worker-laid eggs are policed by nest

mates, and it is in the best interest of a queen-laid egg to be distinguishable from worker-laid eggs. It would be beneficial for a larva to emit a signal that reduces the likelihood of policing and reduces worker laying by inhibiting worker ovary development. In this way, brood pheromones may provide a survival benefit when they inhibit worker ovary development, especially if queen larvae also emit the brood signal. This hypothesis predicts that larval pheromones would decrease the likelihood that workers cannibalize queen-laid eggs. It also predicts that worker laid larvae, and perhaps male larvae, would have low levels of larval pheromone production (or qualitatively different pheromone production), whereas queen larvae would have higher production of larval pheromones. Interestingly, the existence of anarchistic bees that can develop their ovaries despite queen and larval pheromones may indicate that some workers have evolved counter-measures for ovary inhibition.

FINAL REMARKS

Herein, I have discussed the evolutionary implications of this dissertation, presenting several hypotheses that address both the evolutionary history and adaptive value of larval pheromones (Table 4.1). I have suggested several avenues of research to test predictions of the various hypotheses, and in several cases, identified areas in which our understanding is severely lacking. A comparative analysis of larval signals would greatly benefit from the chemical identification of larval odors in additional species, and such a data set would enable tests of several hypotheses about the evolutionary origins of larval pheromones.

Table 4.1. Ultimate level hypotheses on the function of larval pheromones

Level of Analysis	Hypothesis Name	Hypothesis
Evolutionary History	Progressive Provisioning Hypothesis	Larval pheromone evolution and elaboration is fascilitated by the evolution of progressive provisioning
	Floral Diet Hypothesis	The overlap of queens and larval pheromones components is a biosynthetic consequence of a common floral diet
	Odor Bias Hypothesis	Components of larval pheromones, such as EBO, evolved to exploit a pre-existing sensitivity to common floral odors.
	One Signal Hypothesis	BP and EBO are subsets of a more complex brood signal
	Extended Phenotype Hypothesis	Brood signals are an extended phenotype of the queen
Adaptive Value	Infection Status Hypothesis	Larval pheromones evolved from cues related to health / infection
	Foraging Tradeoff Hypothesis	Larval pheromones regulate the relative investment of foraging labor into survival and reproduction.
	Worker Policing Hypothesis	Larval pheromones reduce the likelihood that queen-laid larvae will be eaten by workers (i.e., policing)

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Appendices

APPENDIX A

Table A1. Transcriptome assembly quality metrics

			Numbers of Sequences			
		Sample Name	**Original**	**Post-trim**	**Mapped**	**Ambiguous**
Brood pheromone	Nectar	**BP_N_1**	71942514	69588315	63403082	1287687
		BP_N_2	53645693	52563274	41999255	1100554
		BP_N_3	63979027	62920155	57860385	1235911
	Pollen	**BP_pol_1**	67128679	65461862	60045797	1185692
		BP_pol_2	59092018	57197679	52140326	1051331
		BP_pol_3	77241988	76072961	68548475	1360138
(E)-beta-ocimene	Nectar	**Oci_N_1**	53047267	52115474	47656908	872635
		Oci_N_2	74797382	73821652	66540762	1398907
		Oci_N_3	58415925	57707492	52559278	1064042
	Pollen	**Oci_pol_1**	56405749	55754764	50692401	999273
		Oci_pol_2	64490416	63577096	56607577	1141243
		Oci_pol_3	65478974	64260642	57768360	1150908
Control	Nectar	**X_N_1**	41309867	40826462	37365569	770154
		X_N_2	48938453	48074421	43967970	867514
		X_N_3	94642154	94359186	93838615	1757196
	Pollen	**X_pol_1**	72815162	71668126	61864680	1188210
		X_pol_2	90816956	89900488	58430985	1235398
		X_pol_3	69898738	69112638	59770049	1116617
		AVERAGES	65782609	64721260	57281137	1154634
		MEDIAN	64984695	63918869	57814373	1146076

APPENDIX B

Table B1. Assembly quality metrics given as percentages

			Percentage of Sequences		
		Sample Name	**%Post-trim**	**%Mapped**	**%Ambiguous**
Brood pheromone	Nectar	**BP_N_1**	96.7	88.1	1.8
		BP_N_2	98.0	78.3	2.1
		BP_N_3	98.3	90.4	1.9
	Pollen	**BP_pol_1**	97.5	97.5	1.8
		BP_pol_2	96.8	96.8	1.8
		BP_pol_3	98.5	88.7	1.8
(E)-beta-ocimene	Nectar	**Oci_N_1**	98.2	89.8	1.6
		Oci_N_2	98.7	89.0	1.9
		Oci_N_3	98.8	90.0	1.8
	Pollen	**Oci_pol_1**	98.8	89.9	1.8
		Oci_pol_2	98.6	87.8	1.8
		Oci_pol_3	98.1	88.2	1.8
Control	Nectar	**X_N_1**	98.8	90.5	1.9
		X_N_2	98.2	89.8	1.8
		X_N_3	99.7	99.2	1.9
	Pollen	**X_pol_1**	98.4	85.0	1.6
		X_pol_2	99.0	64.3	1.4
		X_pol_3	98.9	85.5	1.6

AVERAGES		98.34467745	88.26753232	1.766578247
MEDIAN		98.45563433	89.3999626	1.772121682

APPENDIX C

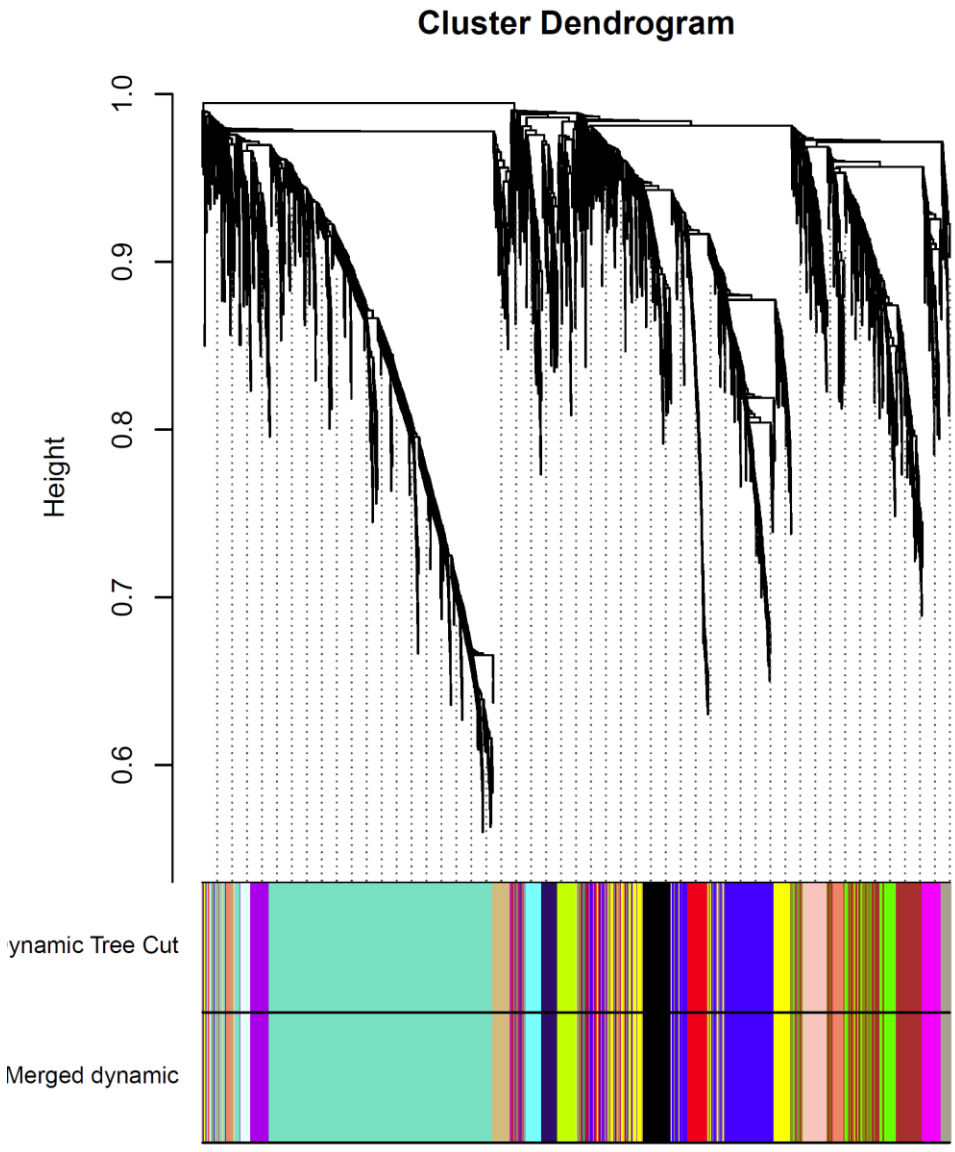

Figure C.1. **Cluster dendrogram of WGCNA modules**

Glossary of Abbreviations

BP – Brood pheromones

DEG – Differentially expressed genes

EBO – (E) – beta – ocimene

FIG - Figure

FDR – False discovery rate

GO – Gene ontology

OCI – ocimene, as in (E) – beta – ocimene

PC – Principal component

PCA – Principal component analysis

X – Control

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Vita

Rong Ma was born in Xian, China. After extensive international experience as an infant, he moved to the United States with his parents, first to Oklahoma, then on to Wisconsin, and finally to California, tracking their graduate and professional careers. After graduation from Poway High School, Poway, California, in 2006, Rong matriculated at Cornell University in Ithaca, New York, where he developed interests in both modern poetry and chemical ecology. He began his research career in the lab of Robert Raguso, working on floral morphology and scent. During the summer of 2009, he was a research intern at the Chicago Botanic Garden. He graduated *cum laude* from Cornell University in May 2010, receiving a Bachelor of Arts degree. During the following two years, Rong taught at the undergraduate and K-12 levels, first as a teaching assistant for organic chemistry at Weill Cornell Medical College in Qatar, and subsequently as a tutor at Sylvan Learning Center, also in Qatar. In August 2012, he moved to Austin, TX to begin a PhD program at the University of Texas with Ulrich Mueller.

Address: rongma.10@gmail.com

This dissertation was typed by the author.