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CHEMICAL COMMUNICATION AND GENOMICS OF SWARMING BEHAVIOR IN HONEY BEES (*APIS MELLIFERA* L.)

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ABSTRACT

Honey bees (*Apis mellifera* L.) are an outstanding model system in which to examine the communication systems underlying complex social behavior. Swarming behavior is a fascinating example of this communication on a grand scale: when honey bee colonies swarm, a large group of workers will depart from their hive with the old queen, leaving behind the rest of the workers and a number of developing queens. The chemical communication systems regulating swarming behavior, and the neurophysiological and molecular mechanisms underlying the responses to these chemical signals and/or cues, have not been characterized. In Chapter 2, we used solid-phase microextraction (SPME) and gas chromatography/mass spectroscopy to examine the volatile chemical compounds produced by the queen during different phases of the swarming process (in-hive, pre-bivouac, and liftoff). We demonstrated that queens release a significantly more complex blend at swarm liftoff than during the previous phases of the process, and that volatiles produced by swarming queens during liftoff are significantly more attractive to workers than volatiles produced by non-swarming queens. We identified five compounds from this blend, one of which is (E/Z)-beta-ocimene, a compound found in mated queens. There was considerable variation among the queens. This suggests that blends of chemicals, rather than a specific chemical, are associated with the swarming process. In Chapter 3, we examined the physiological and brain gene expression differences between workers that remain in the hive versus those that depart with the swarm. Microarray and quantitative real-time PCR analysis demonstrated that these two groups of bees have distinct gene expression patterns, including significantly higher levels of the egg-yolk and storage protein vitellogenin, which is a marker of the nursing behavioral-physiological state. We also identified 142 transcripts that were consistently, significantly, differentially expressed in the brains of swarming versus non-swarming workers from two different colonies. This gene list overlapped significantly with a list of differentially expressed genes in nurses versus foragers. Subsequent analysis of directional overlap demonstrated that swarming workers had similar gene expression patterns as younger, nurse bees. However, the reproductive potential of swarming workers (as measured by the number of ovarioles) did not differ significantly from that of non-swarming workers. This suggests that individual fitness benefits do not factor into the likelihood to join the swarm. Our results demonstrate that queens could play a role in triggering the initial swarming event through the release of novel pheromones, and swarming and non-swarming workers represent distinct physiological and behavioral states that likely are differentially responsive to these pheromones.

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Chapter 1

Introduction

The relationship between genes and social behavior ("sociogenomics") has long been of great interest to biologists, and with the development of genomic tools and resources in a broad array of species, we can now begin to unravel the relationship among genes, the brain, the environment, and social behavior (Robinson et al., 2005). This is a complex relationship since environmental influences can bring about changes in brain gene expression, brain function, and social behavior, while genetic variation among individuals results in behavioral variation in response to these environmental cues. Behaviors such as foraging, reproduction, and parental care become social when interactions between individuals in the same species occur in a way that influences behavior, either immediately or in the future. One of the most vital foundations of social behavior is communication (Robinson et al., 2008), as social animals often act cooperatively, which requires coordination (Robinson et al., 2005).

As Robinson et al. outline in their paper:

> "All systems of social behavior share the following features: (i) They are acutely sensitive and responsive to social and environmental information. (ii) This information is transduced within individual organisms by one or more primary sensory pathways. (iii) The transduced neural signals are processed and integrated in specific circuits of the brain via conserved signal transduction and neuromodulatory systems. (iv) The resulting internal state of the animal ultimately controls its behavior." (Robinson et al., 2008)

Despite the vast array of different social behaviors performed by individuals in different species, there is still an underlying structure that is universal to all social life. By understanding the molecular basis of behavior, it will be possible to gain a more comprehensive view of social behavior, as well as a more thorough knowledge of how genes and social behavior influence each other.

In order to explore communication systems and the relationship between genes and social behavior, I will be using honey bees (*Apis mellifera* L.) as a model system. In a honey bee colony there is one queen and tens of thousands of workers that must coordinate their behavior for colony maintenance and survival. Honey bees demonstrate a form of reproduction at the colony level known as swarming, which is an excellent system for examining the genes associated with complex social behavior and how these genes both regulate and are regulated by communication systems. Swarming represents communication on a grand scale, with the activity of thousands of individuals being coordinated. The old queen and approximately 50-75% of the workers leave with the swarm, but the remaining workers stay with the new queen. My thesis research attempts to answer two questions relating to swarming and communication: (i) how is this behavior coordinated, and (ii) why do only some of the workers respond to the swarming signals by leaving to found a new colony?

Evolution of Swarming Behavior

For the honey bee, swarming benefits the colony by allowing it to divide and reproduce. This is important because lone worker bees, which lack the ability to reproduce on an individual level, cannot survive outside the construct of the colony, while the queen lacks the capability to forage, build comb, or feed brood (Michener 1974). Swarming also provides the departing queen with an entourage of workers to assist with comb building, brood rearing, and foraging at the new nest site. However, swarming is not without its costs. It requires huge investments in both time and energy, and survival is never a guarantee for either the swarm itself or the original colony. In temperate climates, it is estimated that only 8-24% of swarms will survive their first winter (Seeley 1978; Morales 1986). Afterswarms, which depart with newly-emerged virgin queens, are even more at risk of failure (Winston 1987). It has been suggested that swarming originated as a migratory behavior in response to unsuitable conditions. This theory explains why the swarm is led by the old queen, not the newly-emerged queen (Ribbands 1964).

Factors Regulating Swarming

The factors contributing to swarming are complex and interrelated. It is generally thought that swarming is triggered by increased colony size, congestion of the brood nest, worker age distribution (skewed to younger workers), and reduced transmission of queen-produced substances, although none of these alone induce swarming (Winston et al., 1991). Indirect effects of colony crowding and congestion, such as increasing temperatures and poor ventilation, could be contributing factors. Swarming is also influenced by environmental and seasonal factors, including weather conditions and periods of growth in the colony's population (Winston 1987).

Colony size encompasses several different aspects—comb area, colony volume, and worker population. However, it is the size of the active population, not the physical size of the nest, which is most important to swarming behavior (Winston 1987). Experiments have shown that some colonies will swarm despite having more nest space than the population can occupy. It is also possible for colonies to become congested yet show no preparation to swarm, demonstrating that worker density and colony size are not sole deciding factors (Simpson 1958).

Congestion of the brood nest includes both the brood and the crowding of adult worker bees in the brood nest area. This congestion occurs when 90-95% of all the brood nest cells contain brood at various stages of development, leaving very few cells open in which the queen may deposit eggs (Winston 1987). Crowding can also be a result of a large number of bees emerging around the same time. These young bees often remain in the brood nest, displacing older bees that would ordinarily be feeding brood. Unable to perform their normal duties, these displaced bees tend to cluster on the edges of the brood nest, further compounding congestion (Simpson 1958).

The distribution of age among workers also influences swarming. Colonies preparing to swarm typically have a low average worker age combined with a high proportion of young workers. Nearly half the workers in a colony are often eight days old or younger when swarm preparations begin. Colonies which show this disproportionate age structure will initiate swarming about 15 days later, although populations at this time are much lower than just prior to swarming. It has also been suggested that age distribution may only be important when there are already enough workers present in the population for swarm preparation to be started (Winston 1987).

Colony size, brood nest congestion, and worker age distribution are all influenced by resource abundance. Without enough available pollen and nectar resources, colonies cannot grow to the proper size and level of congestion to swarm. Most swarming takes place in mid-spring, usually in May or early June, which corresponds with the abundance of resources in a temperate climate (Winston 1987).

Reduced transmission of queen-produced substances, which is directly related to colony congestion, also plays a role in inducing a colony to swarm. There is no significant difference in the amount of pheromone produced by queens from colonies that are preparing to swarm versus those that are not—it is the transmission of these substances that is reduced (Winston 1987). As the population increases and the colony becomes more congested, transmission of queen pheromones decreases (Naumann et al., 1993). Since these pheromones inhibit certain worker behaviors, such as queen rearing and ovary development, reduced transmission leads to lessened inhibition of these behaviors (Winston et al., 1991). These behavioral and physiological changes in workers are important aspects of the beginning of swarming.

The Swarming Process

Swarming is preceded by a significant increase in oviposition until about a week before the swarm leaves the nest (Winston 1987). The queen's retinue of workers constantly offers food, and this frequent feeding promotes increased egg laying. After several weeks, large numbers of bees begin emerging, creating a population "explosion". At this time, a large proportion of the adults in the colony will be very young bees (Gary 1992). Theoretically, physiologically younger bees are more beneficial to a swarm than physiologically older bees. At the new nest site, most of the workers will be occupied constructing new comb and feeding brood (tasks normally performed by younger bees). No new bees will join the colony until workers begin to emerge 21 days after eggs are first laid by the queen in the new nest, and thus bees with a longer life expectancy are critical (Zeng et al., 2005).

The first visible sign that a colony is preparing to swarm is the appearance of new queen cups, specially shaped comb cells in which queens are reared. Queen cups appear when the colony crosses the threshold of 2.3 worker bees/ml hive volume (Lensky and Slabezki 1981). Colonies

seal, on average, a total of 15-25 queen cells before and immediately after swarming. Swarm liftoff usually occurs just after the first of these cells is sealed, around 8-10 days after queen rearing first begins. This helps ensure that the remaining colony will have at least one virgin queen emerge after the swarm has departed (Winston 1987).

All workers engorge themselves with honey for around 10 days prior to swarming, a behavior that is possibly stimulated by queen rearing. This ensures that they are carrying adequate honey reserves when the swarm departs. The extended period of time over which engorging occurs is likely beneficial, since the exact day and time swarming will actually occur is unpredictable. These honey reserves represent around 40% of the total worker weight of the swarm, and serve as food while in transit and during the first few days in the new nest (Winston 1987). The average weight and sugar concentration of stomach contents of workers from swarming colonies have been found to be significantly greater than those of workers from non-swarming colonies. It is interesting to note, however, that there is no significant difference between those bees that depart with the swarm and those that remain in the colony. This demonstrates that honey engorgement is not responsible for determining which bees depart with the swarm and which bees remain (Combs 1972). Drones do not engorge in preparation for swarming. They have a limited capacity as a source of carbohydrate reserves for the swarm because they are present only in small numbers in a swarm and their honey stomach capacity is less than that of a worker. Drones have a diurnal feeding rhythm, so the amount of honey reserves they are able to contribute to the swarm is dependent on the time of day the swarm departs. Drones' maximum honey stomach contents generally correspond to the start of afternoon flight (Burgett 1973).

Approximately a week before swarming, workers begin to feed the queen less. As a result, her egg laying slows, her ovaries shrink, and her abdomen begins to decrease in weight (Taranov and Ivanova 1946; Allen 1955, 1956, 1960). It is crucial for the queen to lose weight so that she is able to fly with the swarm. It is also important to note that this decreased oviposition is a result, not a cause, of queen rearing in swarming colonies (Simpson 1958). Typically, young, recently-emerged bees would be engaged in brood feeding behaviors. However, because of the sharp decrease in egg laying by the queen, many young bees in the colony are displaced from their usual behavior. These bees begin to fill the hive and are often referred to as "active swarm bees" because they make up the majority of the workers that will leave with the swarm (Gary 1992). The inactivity of the swarm bees plays an important part in the swarming process, and has been

associated with full development of the pharyngeal glands and ovary enlargement. The ovaries will serve as additional protein stores which will facilitate the establishment of the swarm in its new nest. However, this inactivity also comes at the expense of foraging and brood rearing in the parent colony (Ribbands 1964).

During the few hours directly preceding the departure of the swarm, the rate of dorsoventral abdominal vibrations drops and some of the workers begin running back and forth in waves while buzzing in order to excite the other workers (Esch 1967). Scout bees begin producing high-pitched piping sounds, which helps prepare the other workers for take-off. Piping bees will burrow into the swarm cluster and press their thoraces against non-piping bees while producing flight muscle vibrations, stimulating the quiet bees to warm their flight muscles. Piping behavior begins an hour or more before the swarm departs, gradually increases, and peaks during departure. The "buzz-run" signal, on the other hand, first appears shortly before the swarm leaves and is only produced strongly during the departure process. Around 10 minutes before the swarm takes off, the piping bees begin running through the cluster and buzzing their wings in bursts, which is known as "buzz-running". It appears that the small number of bees that produce this buzz-run signal trigger the mass exodus of the swarm from the old nest site (Rangel and Seeley 2008). It has been suggested that this particular signaling system evolved in honey bees because only the scout bees, which are much more mobile than the majority of the colony, are able to sense when all the bees in the swarm cluster are ready for departure. It is possible that, as the scouts move through the cluster while piping and stimulating flight muscle warm-up, they are taking a "census" of the temperature of the swarm. Once they have determined that all of the bees have reached the necessary temperature for take-off, signals such as piping and buzz-running allow them to share this information with the rest of the swarm (Rittschof and Seeley 2008). It is not certain what role, if any, the queen plays in triggering this departure. It has been observed that the queen does not lead the swarm from the hive, but is often pushed out by the worker bees (Simpson 1958).

After the workers and their queen have departed the original colony, they soon form a bivouac at a temporary site near the parental hive. Scout bees then begin the nest site selection process, flying from the swarm cluster to search for potential nest sites. Upon return to the cluster, the scouts report their findings by performing waggle dances (Seeley and Buhrman 1999). The swarm then chooses one nest site from a sampling of five or more possible sites, although it is

still unknown how the scout bees sense a quorum (Seeley et al., 2006). Once the quorum threshold is reached for a single site, the scouts stimulate the workers in the swarm cluster for flight by producing a piping signal (Seeley et al., 2006). The swarm then lifts off again, and the scout bees "streak" through the airborne swarm cloud in the direction of the new nest site, visually indicating the direction of travel (Beekman et al., 2006). This task is crucial, as less than five percent of the swarm bees have visited this new nest site to be aware of its location (Seeley and Buhrman 1999). When the swarm reaches the nest site, scouts will settle at the nest entrance and begin releasing pheromones from their Nasonov glands, which attracts the rest of the workers to the entrance (Ambrose 1976; Seeley et al., 1979).

Pheromones Involved in Swarming

Pheromones produced by both the queen and the swarming workers seem to play a role in regulating swarming. Chemical extracts from several glands in the queen (mandibular, tarsal, and tergite glands) both inhibit queen rearing prior to swarming (Free 1987, Blum 1992), and help stabilize the swarm cluster (Blum 1992). Nasonov pheromone, a substance produced by workers, works in conjunction with queen pheromone (see below for detailed discussion of specific identified components of queen pheromone) to help regulate the movement and formation of the swarm (Blum 1992). It is also used once the swarm has arrived at the new nest site when scouts land at the entrance and release Nasonov pheromone to attract the rest of the swarm to the entrance of the new nest (Seeley et al., 1979; Winston 1987). A deficiency in the blend of the chemicals produced by the queen is thought to initiate the first step in swarm preparation, the construction of queen cups. Possible explanations for this deficiency are that the distribution of pheromones among workers is less efficient due to crowding, an increased amount of pheromone needed (due to worker population increase), or there is an increased threshold in an individual worker's pheromone response prior to swarming. Workers must physically contact the queen in order for queen rearing to be inhibited, suggesting that these pheromones are most likely perceived and transmitted by workers' antennae. This could account for less efficient pheromone distribution, or an increase in the amount needed to inhibit behavior, as the population increases (Free 1987). One study suggested that population size has more effect on pheromone transfer than crowding, and that interference with pheromone transmission may only occur at very high levels of congestion (Naumann et al., 1993).

One early experiment by Butler (1960) measured and compared quantities of "queen substance" among queens of varying types: mated laying queens from colonies with no queen cells, mated queens that had been superseded, and mated queens from swarms. He suggested that queen rearing in overcrowded colonies preparing to swarm is not necessarily due to queen substance production, but rather inefficient collection and distribution (Butler 1960; Winston et al., 1991; Naumann et al., 1993).

The most complete studies have been performed on the chemicals produced in the queen's mandibular glands (queen mandibular pheromone, or QMP), which has five main components: three acids, 9-keto-2 (*E*) – decenoic acid (9-ODA), and R-(-)- and S-(+)-9-hydroxy-2 (*E*) – decenoic acid (9-HDA), and two aromatics, methyl *p*-hydroxybenzoate (HOB) and 4-hydroxy-3-methoxyphenylethanol (HVA). Boch et al. tested live queens, extracts of whole queens, and queen parts, together with synthetic 9-ODA and 9-HDA, for their attractiveness to swarming workers. While 9-ODA and 9-HDA were attractive to workers from queenless swarms, queen extracts were significantly more attractive than 9-ODA, or 9-ODA and 9-HDA. Furthermore, workers were more attracted to extracts of mated laying queens than extracts of virgin queens (Boch et al., 1975).

Thus, previous studies have suggested that pheromone blends produced by swarming queens are more attractive to swarming workers than blends of queens from non-swarming colonies, and levels of QMP are not sufficient to explain these differences. Since queen pheromone is produced by multiple glands, and since swarming is a rapid process that is likely regulated by differences in the release of compounds rather than the synthesis of compounds, in order to identify putative queen-produced swarming chemicals, it is necessary to characterize the full chemical blend produced by the swarming versus non-swarming queen. Recent advances in chemical ecology approaches have increased the feasibility of these studies, with the development of solid-phase microextraction (SPME) technology. This passive volatile collection technique enables non-destructive sampling of volatiles, and can help build a more complete picture of queen pheromone than earlier gland analysis alone could. In 2006, Gilley et al. used SPME to sample queen volatile compounds in unmated, recently mated, and egg-laying queens. They then compared these to volatiles emitted by workers. Out of the nine compounds identified, four were present in queens but not workers. One of these, (*E*)-β-ocimene, was only fully expressed in

established mated queens, suggesting that it may signal diploid egg-laying activity to workers. The other three queen-specific compounds were associated with unmated queens (Gilley et al., 2006).

In Chapter 2, we used SPME technology to examine the complete volatile chemical blends produced by queens in established colonies, resting swarm clusters, and swarm clusters during liftoff. We demonstrated that swarming queens produce significantly more volatile compounds than non-swarming queens. We also showed that swarming workers are more attracted to swarming queens than non-swarming queens.

Molecular and Physiological Mechanisms Underlying Worker Swarming Behavior

Workers have a division of labor related to behavioral maturation, where younger nurse bees perform in-hive tasks, such as comb building and brood care, while older forager bees perform out-of-hive tasks, such as foraging and scouting (Lindauer 1953). This division of labor is regulated by juvenile hormone (JH) levels; increasing levels of JH lead to the transition from nurse to forager (Sullivan 2000). Foraging activity is markedly reduced and JH titers are consistently lower in workers from pre-swarming colonies than from non-swarming colonies (Zeng et al., 2005). There also appears to be a "division of labor" between swarming and non-swarming workers, but the mechanisms underlying this separation are relatively unknown. This could be because swarming workers are more forager-like, and therefore more adept at flying and less of a loss to the colony if they die. It could also be that they are more nurse-like, because nurse bees have greater food stores and can live longer, which is necessary since it will take 21 days before new workers are produced. Furthermore, hypopharyngeal glands (which produce brood food) are significantly larger in nurses than foragers (Crailsheim and Stolberg 1989), and these are also enlarged in workers from pre-swarming colonies relative to control colonies (Zeng et al., 2005). It may be that they are physiologically more nurse-like (in terms of metabolism, fat stores, and longevity) but behaviorally more forager-like (in terms of brain gene expression).

Previous studies suggest that young, nurse-age bees are more likely to depart with the swarm (Butler 1940; Gilley 1998). As noted above, pre-swarming colonies typically have a higher proportion of young bees relative to non-swarming colonies (Gary 1992). However, the swarming

bees are predominantly from the young cohorts of bees, and thus the swarm does not simply reflect the age structure of the pre-swarming colony. This suggests that younger bees are more likely to respond behaviorally to any swarming cues, or swarming occurs during a period in which old, forager bees are simply not present in the hive. Indeed, most swarming occurs during the early morning or late afternoon, when forager bees have left the hive (Butler 1940). The oldest bees in the swarm are generally the nest-site scouts (Gilley 1998). One possible explanation for this is that it is in the swarm's best interest to conserve the younger bees, and scouting may be a risky task. It is also possible that young, less experienced bees are not able to function as well as older bees in nest-site scouting. This suggests that the experience gained from foraging is important in scouting behavior (Gilley 1998). It has been suggested that scouts display "novelty-seeking" behavior, an underlying tendency to seek something new. A recent study identified differentially expressed transcripts between scouts and non-scout foragers, suggesting that scouts are a distinct behavioral class (Liang et al., 2012).

Surprisingly, hemolymph sugar titers (trehalose, glucose, and fructose) and body glycogen reserves are not significantly different in workers from swarm colonies versus those from non-swarm colonies, despite the fact that workers from swarming colonies have filled honey sacs (Leta et al., 1996). There is no significant correlation between the amount of food carried in the honey sac and hemolymph sugar levels in each individual bee, which is most likely due to homeostatic regulation tightly controlling hemolymph carbohydrate levels in honey bees, suggesting that these traits are essentially uncoupled (Leta et al., 1996). Panzenbock and Crailsheim (1997) demonstrated that foragers 28-31 days old have significantly higher levels of glycogen than workers 19 days old. However, this study used age-defined bees, rather than clearly function-defined bees (Panzenbock and Crailsheim 1997). This is important because behavioral maturation and division of labor rely on environmental conditions within the hive, not solely on worker age, so bees of the same age may perform different tasks within the colony. Nurses and foragers differ in their metabolic reserves; nurses have more glycogen. Swarming and non-swarming bees, however, do not differ.

Differences in worker types that depart with the swarm versus those that stay in the colony have also been hypothesized to be linked to individual fitness benefits. For example, if workers are more related to the new queen than the old swarming queen, they may receive greater inclusive fitness benefits by remaining in the colony. However, there is no indication that worker behavior

is influenced by kin selection and relatedness (Breed et al., 1994; Rangel et al., 2009). Alternatively, workers that remain in the colony may have more of an opportunity to activate their ovaries and initiate egg-laying, given that the new queen may not survive, rendering the colony queenless (and thereby allowing worker ovary activation since queen pheromone inhibits this process (Hoover et al., 2003)). Even if the queen does survive, mate, and initiate egg-laying, there will be a lengthy break in the brood cycle that can favor worker ovary activation, since brood pheromone also inhibits this process (Arnold et al., 1994). Indeed, workers completing late stages of larval and pupal development in colonies that recently swarmed have ovaries with more ovarioles (Woyciechowski and Kuszewska 2012), a trait which has been correlated with increased rates of ovary activation (Makert et al., 2006). However, it is unknown if ovariole number in the existing adult population of a swarming colony plays a role in determining which bees swarm; it could be hypothesized that the swarming bees have fewer ovarioles and therefore reduced reproductive potential.

In Chapter 3, we examine these molecular and physiological mechanisms that may regulate the likelihood that a worker leaves with the swarm rather than remaining in the hive. We did not find a significant difference in ovariole number, suggesting that worker reproductive potential/individual fitness does not influence swarming behavior. However, swarming workers had significantly higher levels of *vitellogenin* than non-swarming workers, supporting previous studies that suggest workers that leave with the swarm are more nurse-like. We also identified several hundred genes that are differentially expressed in the brains of swarming and non-swarming workers, allowing us to examine molecular and physiological pathways (including those associated with behavioral maturation and scouting behavior; Alaux et al., 2009; Liang et al., 2012) that are associated with swarming behavior.

Conclusion

In my thesis research, I have examined two questions: (i) how is swarming behavior coordinated, and (ii) why do only some of the workers respond to the swarming signals by leaving to found a new colony? Our results suggest that the queen's volatile chemical blend changes during the course of the swarming process. This indicates that both the queen and her workers play a role in this behavior. We also found significant physiological and transcriptional differences between

swarming and non-swarming workers, suggesting that a distinct group of workers leaves the colony with the old queen to found a new colony. These swarming bees appear to be generally more nurse-like than the bees that remain in the parental nest, which supports previous hypotheses that the younger workers are the ones leaving with the swarm.

Swarming behavior in the honey bee is complex, and is an excellent model for examining the relationship between genes and social behavior. Swarming has evolved as a method of reproduction at the colony level, and demonstrates remarkable coordination between the queen and thousands of workers at the individual level. Genes are both regulating, and being regulated by, communication systems such as the volatile pheromones produced by queens as the swarming process progresses. By understanding complex behaviors such as swarming, it will be possible to gain a more comprehensive view of the causes and consequences of social behavior across taxa.

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Chapter 2

Volatiles produced by honey bee queens change as swarms progress to liftoff

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Introduction

Swarming behavior in honey bee colonies is a fascinating example of collective behavior involving thousands of individuals. The swarming process begins when the colony becomes overcrowded and the workers begin to rear new queens in preparation for colony fission (Winston et al., 1991). Workers start feeding the queen less food, causing her to lose mass and allowing her to better fly with the swarm (Winston 1987). Workers also engorge themselves with honey, and thus carry adequate food reserves when the swarm leaves the parental nest (Combs 1972). Immediately prior to swarming, workers pipe and vibrate the other workers to signal swarm exodus (Rangel and Seeley 2008) and push the queen out of the colony with the swarming workers (Simpson 1958). After the swarm has settled as a bivouac, workers begin the process of searching for and choosing a new nest site (Seeley and Morse 1978). Once a new site is selected, workers again pipe and vibrate other workers, signaling liftoff (Seeley and Tautz 2001).

Most studies have suggested that it is the workers that drive the swarming process, and the queen's contribution is largely passive. However, chemical signals from the queen are critical for coordinating aspects of the swarming process. Reduced transmission of queen pheromones between workers has been linked to the initiation of new queen rearing (Winston et al., 1991), but these reduced pheromone concentrations are thought to be due to colony congestion, rather than a decrease in pheromone production by the queen (Fefferman and Starks 2006). Similarly, queen pheromones help stabilize the swarm cluster while scout bees search for a new nest site (Butler

and Simpson 1967). Swarming workers are extremely sensitive to the presence (or absence) of their queen in an airborne swarm, and will abandon the move and resettle as a bivouac if she is prevented from moving with them (Morse 1963, Simpson 1963). This sensitivity to the queen's presence is likely highly adaptive—without the queen, the swarm will not be able to establish a new colony. Furthermore, the swarm must move en masse to the new nest location, since the queen and majority of workers have not previously visited the site. However, it is unknown if there is a difference in pheromone production between swarming and non-swarming queens that triggers liftoff of the bivouac, or whether queens produce more or additional pheromones to advertise their presence in airborne swarms.

Outside the context of swarming, queens produce a five-component blend of pheromones in their mandibular glands (queen mandibular pheromone, or QMP) that plays a critical role in regulating several aspects of worker behavior, physiology, and colony organization. In addition to stabilizing the swarm cluster (Butler and Simpson 1967), QMP is attractive to workers over short distances (Slessor et al., 1988), inhibits new queen rearing (Winston et al., 1990), reduces ovary development of workers (Hoover et al., 2003), and slows their behavioral maturation (Pankiw et al., 1998). However, several studies have indicated that QMP production can be quite variable, and queen pheromone profiles can be significantly modulated by queen mating state and quality (Kocher et al., 2009). In addition, while previous studies of the role of queen pheromones in swarming have focused primarily on QMP, the full complement of pheromones produced by queens is the product of multiple glands (Keeling et al., 2003), and therefore cannot be completely characterized by studies of any one gland.

Here, we non-destructively characterized volatiles from queens in colonies, queens in bivouacking swarms, and queens during bivouac liftoff, to better understand changes in the chemical signals that queens produce throughout the swarming process. We used solid-phase microextraction (SPME) to collect volatiles from queens. Extracted chemical profiles were examined using gas-chromatography/mass spectroscopy. With this approach, we characterized the full blend of volatiles that are produced by queens (even if they are produced by multiple glands), and identified rapid changes in volatile release that may not be revealed by examining the contents of gland extraction. SPME has previously been used to sample volatiles produced by honey bee queens in different reproductive states (Gilley et al., 2006), volatiles from instrumentally-inseminated versus naturally-mated honey bee queens (Huang et al., 2009), and

volatiles produced by waggle-dancing workers (Thom et al., 2007; Gilley et al., 2012). SPME has also been used to characterize differences in chemical profiles between queens and workers in several other social insect species (ants: Tentschert et al., 2002; wasp venom: Bruschini et al., 2008; termites: Weil et al., 2009).

We compared the volatile profiles of swarming and non-swarming queens to determine: (1) whether queens release greater quantities of volatiles when they swarm; (2) whether queens release different types of volatiles in different phases of swarming; (3) whether the relative proportions of these compounds change as colony fission progresses; (4) whether the chemical blends produced by swarming queens are more attractive to swarming workers than those of non-swarming queens; and (5)initiated identification of specific volatile compounds released by swarming queens.

Materials and Methods

General Bee Rearing

Honey bee colonies were maintained according to standard beekeeping practices at the Wellesley College apiary in Wellesley, Massachusetts, U.S.A. Naturally mated honey bee queens and colonies were purchased commercially in 2010 from Merrimack Valley Apiaries (Billerica, Massachusetts, U.S.A.) and in 2012 from Beehavin Apiaries (Smithfield, Rhode Island, U.S.A.). Queens were primarily of mixed Carniolan (2010) and Italian (2012) descent, and were reared in the year that data were collected from colonies (i.e., less than one year old). Samples of queen volatiles were collected between June 22 and September 2, 2010. Behavioral assays were performed between July 6 and July 27, 2012.

Establishing Swarms

Bivouacking swarms were created using standard methods (Seeley and Buhrman 1999). Briefly, approximately 1-1.5kg of workers from a single colony was shaken off of frames into a wooden box with wire-screen sides (15x25x35cm); the colony's queen was caged and also placed in the

box. Once caged, each swarm was fed a 1:1 sugar/water solution ad libitum for at least six hours per day for two to three days to simulate engorging by workers prior to a natural swarm exodus. After this feeding period was over, the caged queen was removed from the screened box and was hung on a swarm board (Figure 1a). Workers were shaken out onto the base of the stand and allowed to settle on the board in a bivouac around the queen. The swarm was subsequently monitored for preparation for liftoff by observing dancing and listening for piping.

Sampling Queen Volatile Profiles

Volatiles released by queens were collected at three time points for each colony. Initial samples ("in-hive" controls) were collected 24 hours after each queen was caged (but before swarms were shaken into a screened box, so queens were still in the hive) to provide a baseline profile of each queen's volatile production before swarm manipulations. "Pre-bivouac" samples were collected after the feeding period was over and right before a colony was transferred to the swarm stand, so the colony and queen had been "homeless" and clustered for some time, but had not yet begun the house-hunting process. "Liftoff" samples were collected immediately after swarms had dissolved and become airborne to move to their new home. In total, 20 in-hive, 20 pre-bivouac, and 17 liftoff samples were collected. Only 17 liftoff samples were collected due to technical difficulties at the moment of liftoff or inclement weather.

Volatile compounds that were released by queens were sampled using solid-phase microextraction (SPME) fibers—65 μm polydimethylsiloxane divinylbenzene fibers (Supelco SPME fiber 57326U; Sigma-Aldrich, Bellefonte, Pennsylvania, U.S.A.). These fibers were used previously to sample queen volatiles in Gilley et al., 2006 and Huang et al., 2009 and were selected because they captured the largest number of chemicals compared to other tested fibers. Each fiber was prepared for first use by injecting the fiber into a gas chromatograph/mass spectrometer (GC/MS) at 250°C for 30 minutes (according to the manufacturer's instructions). Fibers were also decontaminated before each exposure to a queen by injecting the fiber in a 250°C GC/MS for three minutes and then wrapping the end of the fiber assembly with Teflon tape to minimize adsorption of the fiber with environmental volatiles prior to exposure to queens. The ends of the fiber assemblies were rewrapped with Teflon tape after queen exposure and

before injection of the fibers into a GC/MS. Fiber assemblies were stored in clean glass vials at all other times.

For each sampling, a queen was placed in an unused 50mL glass media jar and then an aluminum-screen cage was inserted into the mouth of the jar before the entire opening was sealed with a double layer of aluminum foil. The tip of a fiber assembly was then inserted into the screen cage in the jar through a small hole that pierced the foil cover (Figure 1b). This setup created a closed chamber for the queen where she could move about freely without making direct contact with the SPME fiber, which passively adsorbs volatiles from within the jar. Before use, all jars and screen cages were cleaned with powdered detergent (Alconox, Inc., White Plains, New York, U.S.A.) and then rinsed thoroughly with deionized water, then acetone, then hexane (and completely dried between rinses). In-hive and pre-bivouac collections were performed with jars completely covered with foil to simulate the darkness of natural colony conditions or being buried within a bivouac. Jars were left uncovered (except for the opening) for liftoff collections to simulate the light conditions that queens would experience in flight.

Each collection jar was sampled empty to generate its "blank" volatile profile before a queen was placed in it. Blank and queen samples were collected from jars for 15 minutes under ambient field conditions and in a shaded area of the apiary. Fibers were injected into the GC/MS as soon as possible and always on the same day that volatiles were collected.

Analyzing Queen Volatile Profiles

All GC/MS data was analyzed using ChemStation software (Agilent Technologies, Inc., Santa Clara, California, U.S.A.). The sensitivity for each chromatogram was set individually in order to include the maximum number of compounds for each sample. Only compounds between retention times of 5 and 16 minutes, where the majority of compounds were detected (>90%), were included in the analysis. Any compounds that were also present in the blanks were removed from the analysis for that sample, resulting in 129 compounds remaining for the analysis. Total volatile production for each sample was calculated as the total area under all peaks that met these criteria. The total number of compounds (peaks) per queen profile was also determined.

We compared the compounds from queen samples across each of the three time points (in-hive, pre-bivouac, or liftoff). A compound was identified according to its retention time (within a window of ±0.02 minutes for each retention time). We then identified "candidate compounds", which were defined as compounds present in at least seven, or approximately 40%, of queens, within a single time point. Individual chemical compounds were identified using ChemStation software and the National Institute of Standards and Technology (NIST) mass spectrometry library. SPME fibers were also exposed to individual QMP compounds: 9-ox-2-decenoic acid (9ODA) (20µL of 1 Qeq), 9 hydroxydec-2-enoic acid (9HDA) (20µL of 1Qeq), methyl-p-hydroxybenzoate (HOB) (20µL of 10 Qeq), 4-hydroxy-3-methoxyphenylethanol (HVA) (20µL of 10 Qeq) as well as both 10 and 20µL of a QMP blend at 10 Qeq (Contech, Victoria, British Columbia, Canada). These were analyzed using the GC/MS to identify their retention times.

Assessing Attraction of Workers to Queen Volatiles

Bivouacking swarms were created from eight colonies using the above methods and were monitored for liftoff. Each swarming queen was paired with a non-swarming queen (control) from a colony that was established in the same year and from the same commercial stock. When a bivouac lifted off, its queen and her non-swarming counterpart were placed in separate collection jars (as previously described). The jar containing the non-swarming queen was covered with foil to simulate in-hive conditions and the jar with the swarming queen was left uncovered to simulate flight conditions. After the jars were exposed to the volatiles of either queen for 15 minutes, the queens were returned to their colonies and the jars were used in an assay that compared the attraction of workers to the volatiles produced by swarming and non-swarming queens.

Approximately 300-400 workers were taken from a bivouacking swarm (different from the swarm containing the focal queen) using an insect vacuum (BioQuip, Rancho Dominguez, California, U.S.A.) and then transferred into a choice-test apparatus (Figure 2). The apparatus was a 10x20x28cm Plexiglas box with a screened lid to provide ventilation and a sealable hole on each short side to which the collection jars could be attached. Once test workers were in the apparatus, it was placed in a shaded area and covered with a dark cloth for at least 30 minutes to give workers time to acclimate to the testing arena.

To start the behavioral assay, a tightly fitting piece of tygon tubing was inserted into the mouth of each glass jar (one containing a swarming queen's volatile profile and the other a non-swarming queen's), the other end of which was tightly attached to one of the holes on the side of the choice-test box. The box and jars were then covered with dark cloth and the workers were permitted to move freely about the box and into either jar. After one hour, each jar was removed, sealed, and chilled, and then the number of workers in each jar was counted. Only the population of workers that made a choice (those that entered either jar) was used for statistical analysis.

Statistical Analysis

Statistical analyses were performed using JMP 10 (SAS Institute Inc., Cary, North Carolina, U.S.A.). Mean total volatile production was analyzed using an ANCOVA with number of peaks per queen as the covariate. Mean number of compounds per queen was compared among the three sample groups (in-hive, pre-bivouac, and liftoff queens) using a one-way ANOVA. A Tukey-Kramer post-hoc test was used to separate means where significant differences were found. Differences in the number of workers attracted to swarming versus non-swarming queens were analyzed using a t-test.

Results

Chemical Analyses

Although the differences were not statistically significant, the total quantity of volatiles generally increased during the swarming process, with the lowest levels found from queens that had not yet begun the swarming process ("in-hive" queens), intermediate levels from queens once colonies had been homeless for several days ("pre-bivouac" queens), and the highest levels in queens that were trying to lift off with a swarm ("liftoff" queens; Figure 3; ANOVA, $F(2,53)=3.06$, $p=0.0554$). However, queens produced significantly more compounds at liftoff than they did when they were in hives or had just begun the swarming process as a new bivouac (Figure 4; ANOVA, $F(2,48)=12.9760$, $p<0.0001$; Tukey-Kramer post-hoc test: IH/L $p<0.001$, PB/L $p=0.0009$, IH/L $p=0.3638$).

In total, 129 unique compounds (not found in the blanks) were identified across all of the samples. However, there was considerable variation in the number of compounds that were produced by each queen (Figure 5), with queens producing between 2-25 compounds when they were in hives, 3- 24 compounds when they were clustered and about to begin house hunting, and 9-39 compounds at the moment of liftoff. To reduce the complexity of the data sat and focus on compounds that were most commonly present, we screened for compounds that were produced by at least seven queens in a single phase of swarming (~40% of the queens in a sample group). Based on these criteria, 17 "candidate compounds" were identified and used for subsequent analysis (Figure 6). A total of five of these compounds were chemically identified using the NIST library. These compounds were benzyl alcohol (retention time 8.39 minutes); (E/Z)-3,7-dimethyl-1,3,6-octatriene ((E/Z)-beta-ocimene; RT 8.52); (E)-6,10-dimethyl-5,9-undecadien-2-one (geranyl acetone; RT 12.54); pentadecane (RT 12.89); and benzyl benzoate (RT 15.09). None of the 17 candidate compounds had retention times that matched those of the five components of QMP (9-HDA: 7.77 minutes; 9-ODA: 9.28; HOB: 12.71; HVA: 13.10; QMP blend: 9.28).

The 17 candidate compounds were produced by different numbers of queens in the different phases of swarming. Two compounds were produced by fewer liftoff versus in-hive queens (compounds with retention times 7.78 and 8.52 minutes in Figure 6), while the other 15 compounds were produced by more liftoff versus in-hive queens. For 10 compounds, pre-bivouac queens had intermediate production of these compounds (compounds with retention times 7.78, 9.12, 9.88, 10.16, 11.13, 11.23, 11.90, 12.04, 12.14, and 12.89 minutes in Figure 6).

Behavioral Assays

Workers were significantly more attracted to the volatiles that were produced by swarming queens than non-swarming queens (Figure 7; t-test; $t(14)= -2.2040$, $p=0.0224$). There was considerable variation across replicates in the total number of workers that left the choice-test apparatus's central box to enter a jar, which could be due to differences inherent to each replicate in the test workers, the paired queens, or environmental conditions at the time of testing.

Discussion

By sampling the total airspace volatiles that are produced by the queens as colonies move through the swarming process, we demonstrated that queens in the swarm bivouac and during swarm liftoff tend to produce greater quantities of volatile compounds than in-hive queens sampled from non-swarming colonies. Queens also produced a significantly greater number of volatile compounds at the moment that bivouacs became airborne than the queens did when they were in hives (before the swarming process began) or when they were clustered in bivouacs in stationary swarms. Workers from swarms were significantly more attracted to the volatiles that were produced by queens at liftoff than queens that were in hives and had not been swarming. These changes in queen volatile profiles with the onset of liftoff of a swarm strongly suggest that queens modify their production of chemical signals to either stimulate swarm liftoff and/or inform workers of their presence as they fly together to a new nest site. This information is critical for ensuring the success of the swarming effort, which would fail were the swarm to lose its queen in transit. While previous studies of changes in queen pheromone production have focused on differences between virgin and mated queens (Slessor et al., 1990; Gilley et al., 2006), our results suggest that, like workers, mated queens can alter their chemical output based on relatively short-term changes in physiological, social, or environmental state. These results also suggest that queens play a more active role in organizing the swarming process than was previously known. However, it remains to be determined the nature of the stimuli that triggers the release of these additional compounds from queens during swarm liftoff: this change may be due to signals from the workers, such as piping the queen (Pierce et al., 2007), or other factors, such as an increase in the temperature and activity level of bivouacking workers as the swarm prepares to lift off.

There was considerable variation among the queens, both in terms of total volatile output and in terms of the total number of compounds produced. Honey bee workers are capable of distinguishing their own queen from others, and newly introduced queens are often executed (Breed 1981). Therefore, these differences in chemical profiles may be associated with individual variation and queen-specific recognition cues. It must be noted, however, that in the behavioral assays, workers from a different colony were still significantly more attracted to the volatiles produced by a swarming queen relative to a non-swarming queen, suggesting that the overall changes in chemical profiles are still attractive regardless of variation among individual queens and colonies. Furthermore, there was no single compound that was found in all of the liftoff

queens, which suggests that it is a blend of components rather than a single compound that is behaviorally relevant. However, two unidentified candidate compounds (compounds 5 and 8) were not found in any queens prior to swarming, and thus may be specifically associated with swarming. The limitations of the SPME sampling method used must also be taken into consideration, as no single type of SPME fiber can adsorb 100% of the volatiles produced by a queen.

One of the candidate compounds was identified as (E/Z)-β-ocimene by mass spectroscopy. (E)-β-ocimene was identified in queen volatiles by previous studies (Gilley et al., 2006; Huang et al., 2009). Levels of (E)-β-ocimene are significantly higher in egg-laying queens than in unmated queens, and the levels of this compound appear to be linked to ovary activation and ovipositioning (Huang et al., 2009). Interestingly, levels of (E/Z)-β-ocimene were significantly lower for queens at liftoff compared to queens sampled when they were in hives. Since swarming queens have reduced egg production in their ovaries (Winston 1987), this suggests that ocimene production could be strongly associated with this process. The other four identified compounds were not previously described in studies of queen chemical production (Gilley et al., 2006; Huang et al., 2009). All of these compounds were found in queens before swarming began, and only one of these compounds (pentadecane) was differentially expressed by the queens as swarming progressed (i.e., significantly higher in liftoff queens versus in-hive queens).

None of the five components of QMP were identified in our study. Previous studies suggested that 9-ODA, a component of QMP, is important for stabilizing swarms (Butler et al., 1964). It is possible that the QMP components play a role in the swarming process, but these chemicals were not released at high enough concentrations to be readily detected by our SPME sampling method. Indeed. we detected strong signals from the individual compounds and QMP synthetic blend only when high concentrations (20 queen equivalents) were sampled, with a queen equivalent defined as the amount of the compound present in the gland (Keeling et al., 2003). It is possible that the type of fiber we used (polydimethylsiloxane divinylbenzene) does not readily adsorb these compounds, or that these compounds simply have low volatility; QMP is typically considered to be a contact pheromone for workers (Slessor et al., 2005). However, any signal used by the queen during swarming must be relatively volatile and/or produced at high quantities in order to provide adequate information to workers in an airborne swarm of their presence in (or absence from) the swarm. Furthermore, workers would be expected to be extremely sensitive to these chemicals,

considering that individuals in swarms have no direct contact and swarms occupy a large volume of open, often windy, space (Morse 1963; Seeley et al., 2008). That such chemical signals exist is strongly suggested by workers' abandonment of a swarming attempt if queens are prevented from traveling with a swarm (Morse 1963, Simpson 1963). However, it must be noted that the main component of QMP, 9-ODA, acts as a long-range sex attractant during queen mating flights (Brockmann et al., 2006), so chemicals that are passed within colonies by contact may also function as signals in open-air situations where communication is required between castes. This suggests that while drones are substantially more sensitive to 9-ODA than workers (Brockmann 1998), queens may also produce substantially greater amounts of 9-ODA during mating flights to attract drones than when swarming.

Swarming is a fascinating model of collective behavior. While previous studies have focused primarily on the role workers play in initiating swarm preparations, stimulating liftoff from the established colony and the bivouac, and scouting for new nest sites (Winston et al., 1991; Seeley and Buhrman 1999; Seeley et al., 2006; Rangel and Seeley 2008; Rittschof and Seeley 2008), it is clear that the queen is not totally passive in the swarming process. Although the queen is unaware of where the swarm plans to settle, and has no influence over the speed of the colony fission process, it is critical that she accompanies swarming workers each step of the way or the swarming process will fail. For this reason, it is crucial for workers in a swarm to be aware of the queen's presence (or absence), and queens are presumably under heavy selection pressure to evolve signals that accomplish this as everyone moves together through the open air, the most dangerous and unpredictable place a colony can find itself, and toward their new home. Our results provide the first evidence that the volatile blend produced by the queen does change during the course of the swarming process, and the workers respond behaviorally to these changes. Thus, both the individual (the queen) and the group (the workers) play a role in this collective behavior. Further studies are necessary to understand the mechanisms underlying the production of these altered blends, which factors trigger it, and if the blend serves simply to confirm the presence of the queen or to actively alter the behavior of the swarm.

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Figures

Figure 2-1. Experimental setup. (a) Swarm board. (b) SPME collection setup.

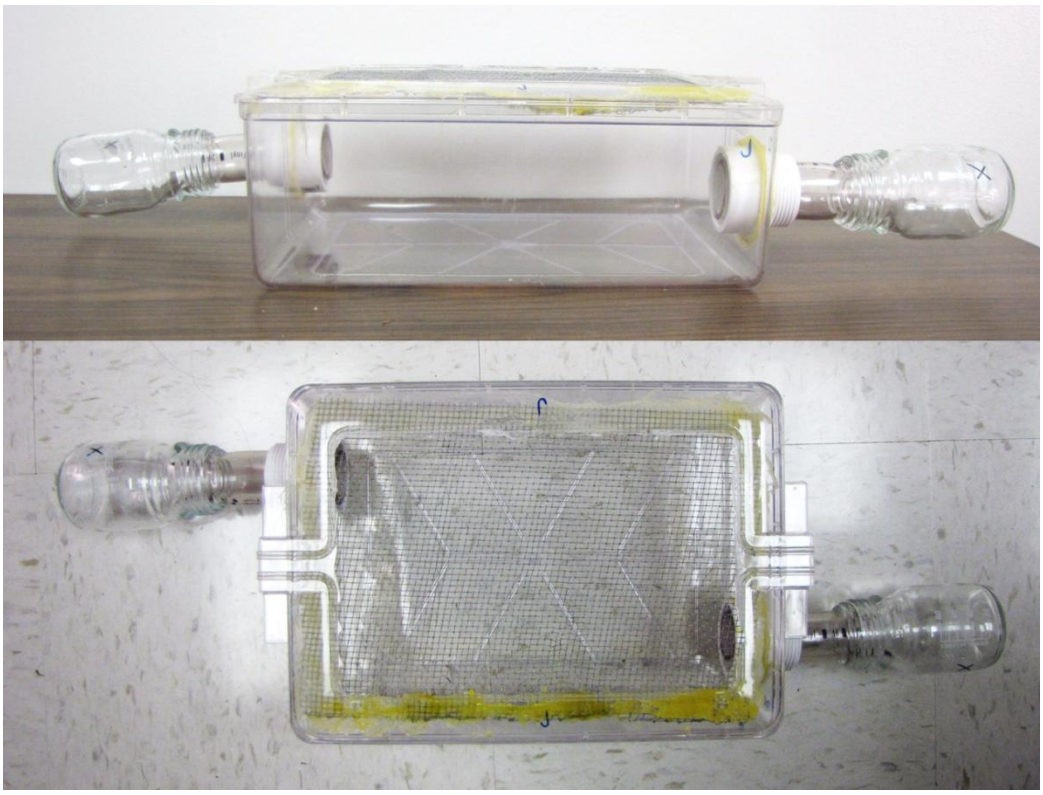

Figure 2-2. Choice test apparatus. (above) Side view. (below) Top view of screened lid.

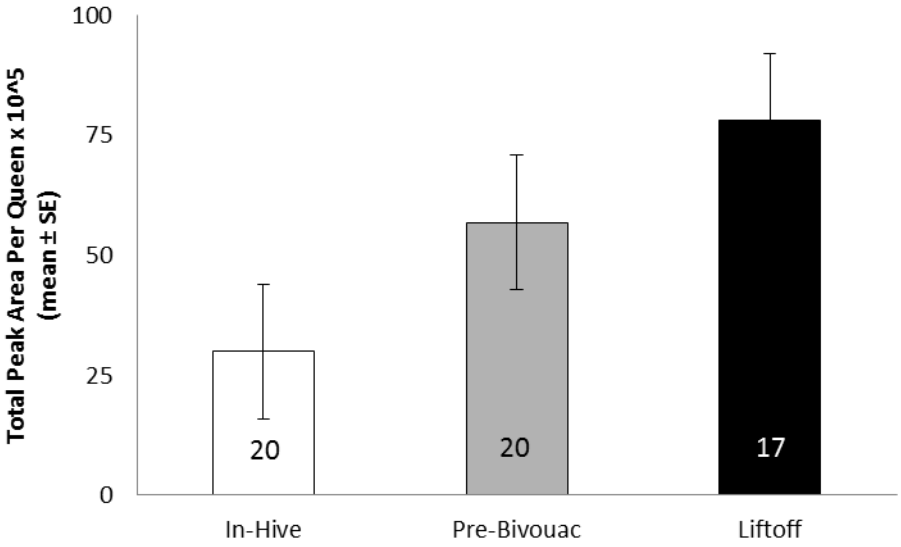

Figure 2-3. Total volatile pheromone production. The total quantity of volatiles produced by each queen was calculated as described in the methods. Queens produced increasing quantities of volatiles as the swarming process progressed, but this difference was not significant. ANOVA; $F(2,53)=3.06$, $p=0.0554$. The total number of queens in each category is represented in the bottom of the bar.

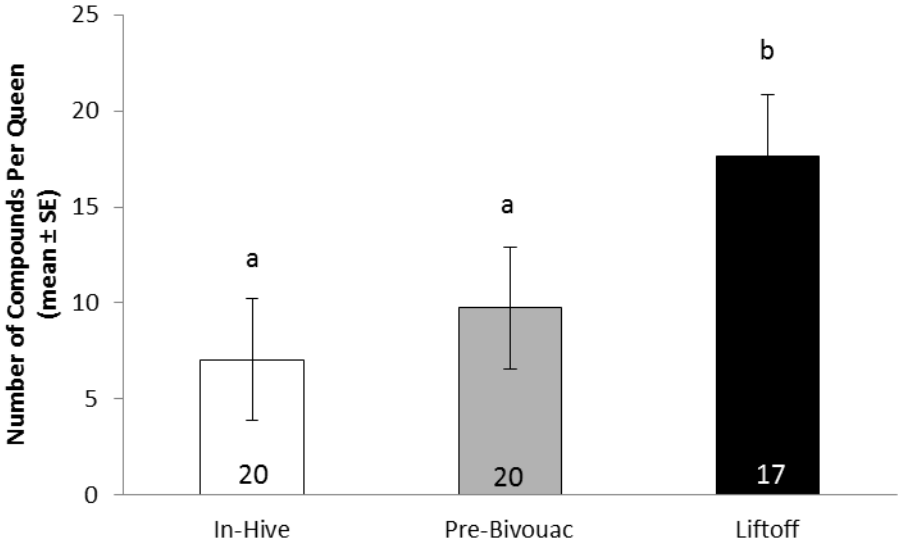

Figure 2-4. Total number of compounds produced. The number of compounds produced by the queens was determined as described in the methods. Queens produce significantly more compounds at liftoff than before swarming. ANOVA; $F(2,48)=12.9760$, $p<0.0001$; Tukey-Kramer post-hoc test, IH/L $p<0.001$, PB/L $p=0.0009$, IH/L $p=0.3638$. Sample points with different letters have significantly different means ($p<0.05$). The total number of queens in each category is represented in the bottom of the bar.

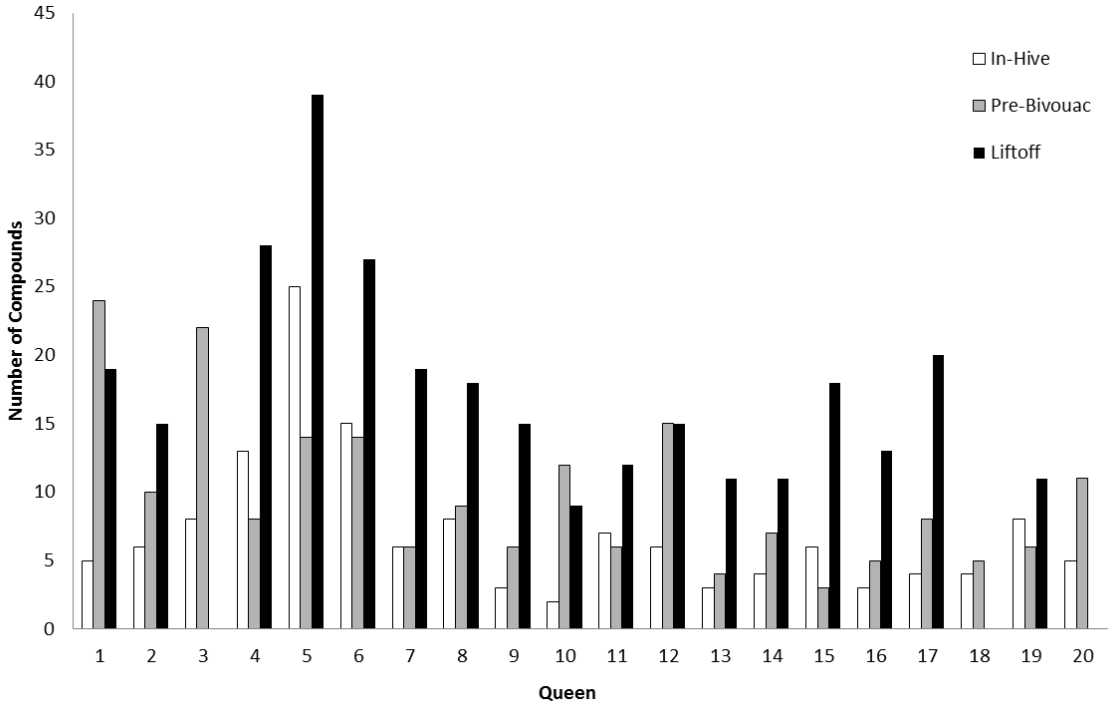

Figure 2-5. Number of compounds produced by each queen. There was substantial variation in the number of compounds produced by each queen. Samples were not collected from queens 3, 18, and 20 during liftoff because of technical issues.

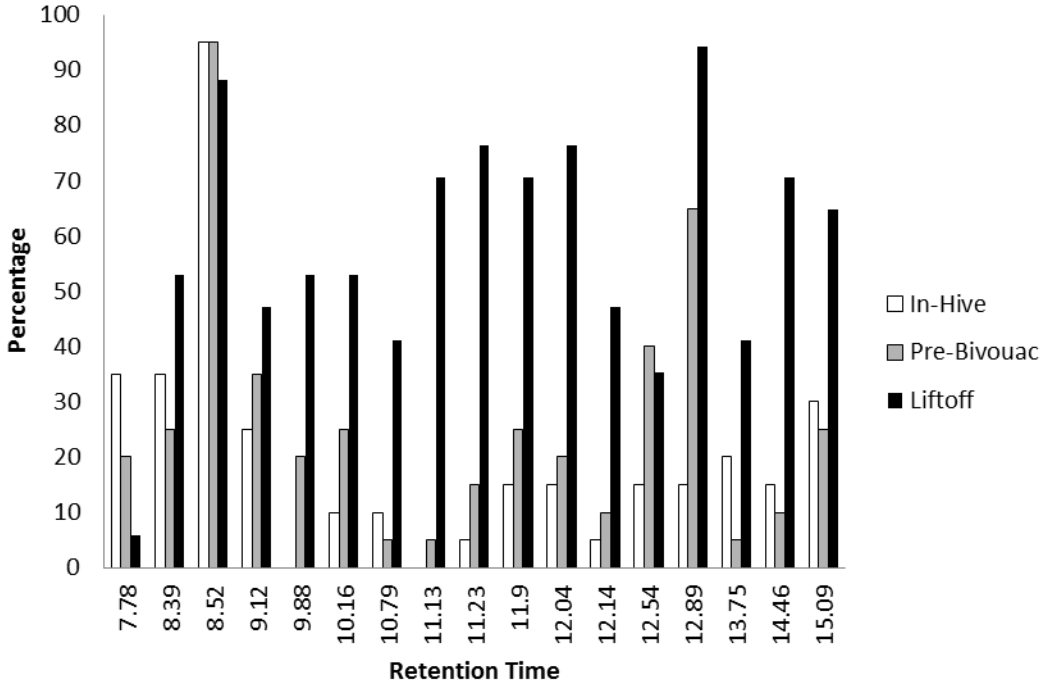

Figure 2-6. Percentage of queens producing each candidate compound. Candidate compounds were identified as described in the methods. The percentage of queens producing each of these compounds varied among compounds and across phases of the swarming process.

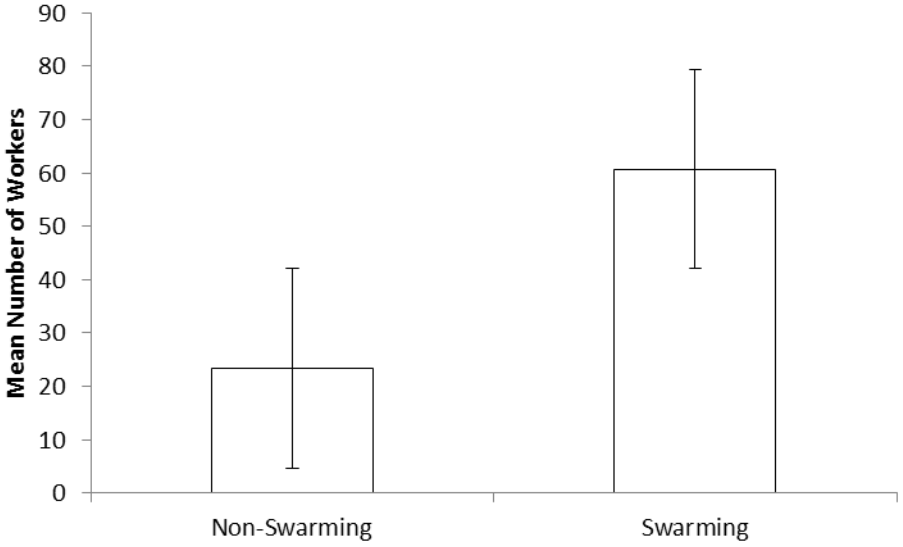

Figure 2-7. Attraction of workers to the volatiles of swarming and non-swarming queens. Workers were significantly more attracted to swarming queens than non-swarming queens. t-test; t(14)= -2.2040, p=0.0224.

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Chapter 3

Should I stay or should I go? Genomic analysis of swarming behavior in honey bees

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Introduction

A hallmark of eusocial insect societies is reproductive division of labor, in which one or a small group of dominant individuals (queens) reproduce while the remainder of the individuals (workers) assist with rearing their offspring (Wilson 1975). In many social groups, colonies are further organized by division of labor amongst subordinate individuals, where workers specialize in various non-reproductive tasks that support colony function (Fewell et al., 2009). Sometimes task performance is determined by the physical traits of workers (e.g., soldier ants are larger and better armed than their non-soldier counterparts), but often labor is divided amongst workers that are physically similar. In the latter case, there is considerable interest in determining the molecular and physiological mechanisms that mediate division of labor amongst subordinates. This is particularly true of honey bees, where all workers are monomorphic and tasks are allocated among many behavioral castes (reviewed in Smith et al., 2008). For the most part, division of labor in honey bees has been studied primarily in established, stable social groups (Toma et al., 2000; Toth and Robinson 2005). However, honey bee colonies are thrown into a relatively disorderly state when they begin the process of colony fission through swarming. When a honey bee colony swarms, some of the workers remain in the established nest with developing queen larvae, while 50-75% of the workers (Rangel and Seeley 2012) and their mother queen leave the colony en masse to found a new nest in another location (reviewed in Seeley 2010). Swarming is risky in both the short and long term: homeless swarms are vulnerable while they house hunt, and once established in a new nest site, they must race against the clock to build food

reserves to last them through seasonal dearths. Most swarms (75% or more) fail at this attempt (Seeley 1978; Morales 1986). Because of the energetic demands that are placed on the fixed population of a newly founded colony, the molecular and physiological traits that underlie the decision to depart the parental nest with a swarm (or to stay behind) are of great interest.

There is some evidence that worker division of labor in established honey bee colonies may play a role in determining who leaves the colony and who stays when swarming begins. Honey bee workers undergo a behavioral maturation process, where they specialize in brood care when they are young (nurse bees) and transition to foraging behavior as they mature (Seeley 1982; Smith et al., 2008). Previous studies have suggested that the younger bees are more likely to depart with the swarm (Butler 1940; Gilley 1998). Most swarming occurs during the early morning or late afternoon, when forager bees have left the hive (Butler 1940). This demographic division is likely adaptive because younger bees should be in a better physical state for the demands of successfully establishing a new colony: they have greater fat stores (Toth and Robinson 2005) and a longer remaining lifespan than older workers, which should make them more likely to survive until the first cohort of workers are reared to adulthood in the new nest (at least 28 days after leaving the parental nest; Zeng et al., 2005). While it may be beneficial to have physiologically younger bees, it could also be argued that swarming bees show forager-like behavior because they readily leave the parental nest, they fly long distances, and a minority of them participate in the search for and reporting of potential nest sites. Further complicating the analysis is the possibility that workers who remain in the parental nest differ in reproductive potential from those who leave with their mother queen. From just prior to the departure of the swarm until the new queen begins to lay eggs in the colony that she inherited, workers who stay behind undergo a one to two week period without a laying queen (or total queen loss if the virgin queen is lost on her mating flight). Because queen and brood pheromone inhibit the activation of workers' ovaries (Butler 1957; Arnold et al., 1994; Hoover et al., 2003), a post-swarm colony may provide ideal conditions for workers to activate their ovaries. Levels of worker ovary activation are high early in the summer when swarms are most likely to issue from colonies (Hoover et al., 2006), and are known to increase in adults and larvae that are exposed to queenless conditions after a swarm leaves its parental nest (Kropacova and Haslbachova 1970). Thus, workers with low reproductive potential may preferentially join the swarm, while workers with high reproductive potential may decide to remain in the nest with the developing queens.

Here, we make the first-ever attempt to characterize the physiological and molecular differences between workers that join swarms versus those that stay behind in colonies in relation to known correlates of division of labor, including nursing, foraging, and reproductive state. We do this by (1) determining whether there are differences in the reproductive potential of swarming and non-swarming workers; (2) determining whether there are differences between swarming and non-swarming workers in levels of *vitellogenin*, a protein that is strongly differentially expressed by nurses and foragers (Fischer and Grozinger 2008); and (3) characterizing genome-wide differences in brain gene expression between swarming and non-swarming workers.

Materials and Methods

Collecting Samples from Colonies

Honey bee colonies were maintained according to standard beekeeping practices at the Wellesley College research apiary in Wellesley, Massachusetts, U.S.A. Colony #1 was headed by an Italian queen (derived from *Apis mellifera ligustica* stock purchased from Reseska Apiaries, Inc., Holliston, Massachusetts, U.S.A.). Colony #2 was headed by an Italian queen purchased from Merrimack Valley Apiaries (Billerica, Massachusetts, U.S.A.). Both colonies were permitted to progress naturally toward a swarming event.

Colonies were monitored every 2-3 days during early spring 2010 for signs that they were getting ready to swarm (e.g., crowded conditions, absence of eggs, the queen's abdomen appeared smaller, presence of queen cells with larvae in them). Once it became clear that a colony was getting ready to swarm, an observer monitored it each morning and afternoon until it finally swarmed, listening for an increase in piping from within the colony (using microphones that were inserted into the colony, according to Rangel and Seeley 2012). Once workers began their rapid exodus from the hive, they were collected with an aspirator directly onto dry ice from both the swarm cluster and from inside the original colony. All samples were then stored at -80°C and shipped on dry ice to the Pennsylvania State University for processing, where they were stored at -80°C until the samples were processed. Samples from Colony #1 were collected May 10, 2010. Samples from Colony #2 were collected July 13, 2010.

Dissections and RNA Extractions

Workers were dissected in chilled RNAlater (Qiagen, Valencia, California, U.S.A.). The ovarioles from both ovaries (when possible) were counted. Statistical analyses were only performed when ovariole counts from both ovaries could be obtained. T-tests to examine mean differences between ovariole counts for swarming and non-swarming workers were performed using JMP 10 (SAS, Cary, North Carolina, U.S.A.); each colony was analyzed separately.

The eviscerated abdomens and fat bodies from sampled workers were stored in grouped pools of five at -80°C. Individual heads were freeze-dried and the brains dissected out on dry ice; the brains were then stored in pools of five at -80°C. Eight pools of fat bodies from five bees from each of the two sample groups (80 workers total per colony) and four pools of brains of five bees from each sample group (40 workers total per colony) were dissected for Colony 1 and Colony 2.

RNA was extracted from each pooled group of fat bodies and brains using homogenization in TRIzol (Life Technologies, Grand Island, New York, U.S.A.) followed by extraction of RNA (with an RNeasy RNA extraction kit; Qiagen, Valencia, California, U.S.A.) with an additional DNase step (RNase-Free DNase Set, Qiagen, Valencia, California, U.S.A.). Sample quality and quantity was verified using a Nanodrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, Delaware, U.S.A.). RNA that was extracted from each pooled group was then stored at -80°C.

Quantification of *vitellogenin* RNA Levels

Quantitative real-time PCR (qRT-PCR) was used to analyze the levels of *vitellogenin* RNA that were present in the eviscerated abdomens and fat bodies of the workers of each sample group. cDNA was synthesized from 200ng RNA using SuperScript reverse transcriptase (Life Technologies, Grand Island, New York, U.S.A.). Expression levels of *vitellogenin* were measured with an ABI 7900 (Applied Biosystems, Foster City, California, U.S.A.) and the SYBR green detection method (Applied Biosystems). The genes *actin* and *eIF-S8* were used as controls to assess expression of *vitellogenin* relative to these housekeeping genes. Primers for *actin* were used as in Grozinger et al. (2003), primers for *eIF-S8* were used as in Richard et al. (2007), and

primers for *vitellogenin* were used as in Fischer and Grozinger (2008) (see sequences in Table 1; primers were ordered from Invitrogen, Life Technologies, Grand Island, New York, U.S.A.). For each sample, triplicate qRT-PCR reactions were performed. A standard curve was generated for each primer using dilutions of genomic DNA. A negative control (cDNA reaction without RT-enzyme) was used to verify lack of contamination. For each colony, the ratio of the expression level of *vitellogenin* to the geometric mean of the housekeeping genes was calculated.

Statistics for qRT-PCR were performed using JMP 10 (SAS, Cary, North Carolina, U.S.A.). Significant differences in *vitellogenin* RNA levels between swarming and non-swarming workers were analyzed using a two-factor ANOVA with behavior and colony, and their interaction, as effects.

Brain Gene Expression Analysis Using Microarrays

Microarrays were used to identify differentially expressed genes in swarming and non-swarming workers using RNA extracted from five pooled whole brains, with four pools per sample group (swarming versus non-swarming) per colony. RNA was amplified from RNA using the Ambion MessageAMP II aRNA amplification kit (Grand Island, New York, U.S.A.). Each sample was labeled independently with both Cy3 or Cy5 using the Kreatech ULS aRNA fluorescent labeling kit (Amsterdam, the Netherlands) and hybridized with the Maui Hybridization System (BioMicro Systems, Salt Lake City, Utah, U.S.A.) to whole genome, oligonucleotide microarrays obtained from the University of Illinois, Urbana-Champaign. Arrays were scanned with an Axon Genepix 4000B scanner (Molecular Devices, Sunnyvale, California, U.S.A.) and viewed with GENEPIX software (Agilent Technologies, Santa Clara, California, U.S.A.). A loop design was used, resulting in 16 arrays (two sample groups, two colonies, four replicates per sample).

For analysis of the data, spots with an intensity less than 100 (the array background intensity) were removed. Transcripts with fewer than seven observations were also removed. Expression data were log-transformed and normalized using a mixed-model ANOVA in SAS 9.3 (proc MIXED, SAS, Cary, North Carolina, U.S.A.):

$$Y = \mu + dye + block + array + array*dye + array*block + \epsilon$$

where Y is expression, dye and block are fixed effects, and array, array*dye, and array*block are random effects. Differentially expressed genes were detected using a mixed-model ANOVA:

$$Y = \mu + \text{behavior} + \text{colony} + \text{behavior*colony} + \text{spot} + \text{dye} + \text{array} + \epsilon$$

where Y represents the residual from the previous model. Behavior, colony, behavior*colony, spot and dye were fixed effects and array was a random effect. P-values were corrected for multiple testing using a false discovery rate of FDR<0.01 (proc MULTTEST, SAS).

Principle components analysis was performed using JMP 10 (SAS, Cary, North Carolina, U.S.A.). Gene ontology (GO) analysis was performed using DAVID version 6.7 with a cutoff of $p<0.05$ (Dennis et al., 2003; Huang et al., 2009). For all GO analyses, array transcripts were matched to Flybase genes and the entire array transcript list with matches to Flybase genes was used as a background list (the 12908 unique transcripts on the arrays yielded 7076 unique Flybase gene matches).

Gene List Comparisons

Transcripts/genes differentially regulated by swarming/non-swarming were compared with transcripts/genes differentially expressed in scouts versus non-scouts (Liang et al., 2012) and nurses versus foragers (Alaux et al., 2009).Genes that overlapped between lists were identified using Venny (Oliveros 2007) and significance was determined using a hypergeometric test (http://nemates.org/MA/progs/overlap_stats.html).

Results

Ovariole Number

Previous studies have demonstrated that workers with more ovarioles are more likely to activate their ovaries in the absence of a queen (Kropacova and Haslbachova 1970; Makert et al., 2006),

suggesting that workers with greater reproductive potential may be more likely to remain behind in a temporarily queenless colony where egg laying opportunities are increased (Woyciechowski and Kuszewska 2012). However, we found no significant differences in ovariole number between swarming and non-swarming workers (Figure 1; t-test; Colony 1: $t(51)=-0.5690$, $p=0.2860$; Colony 2: $t(35)=0.9710$, $p=0.1691$).

vitellogenin RNA Levels

RNA and protein levels of *vitellogenin* (*Vg*), an egg yolk and storage protein, are significantly higher in nurse bees than foragers (Fischer and Grozinger 2008), and reducing levels of *Vg* with RNAi accelerates the transition to foraging (Nelson et al., 2007). In our study, *vitellogenin* levels were significantly higher in swarming workers than they were in non-swarming workers, with a two- to four-fold increase in *vitellogenin* if workers departed with the swarm (Figure 2; two-factor ANOVA; behavior: $F(1,24)=25.6626$, $p<0.001$; colony: $F(1,24)=1.2403$, $p=0.2764$; behavior x colony: $F(1,24)=3.7591$, $p=0.0644$).

Brain Gene Expression Patterns

At a false discovery rate (FDR) of 0.01, we found 1,808 unique differentially regulated transcripts between swarming and non-swarming bees from the two colonies that we examined, with 859 unique transcripts. Expression changes in the majority of these genes were associated with a colony effect (Table 2) or showed a significant colony x behavior interaction. Only 142 unique transcripts were significantly differentially regulated as a result of a behavior-only effect. A principle components analysis revealed the effects of behavior and colony on gene expression patterns. Colony explained 51.7% of the variability, behavior explained 14.5% of the variability, and a behavior by colony interaction explained 33.8% of the variability (Figure 3).

Gene ontology (GO) analysis of these 142 transcripts (corresponding to 94 unique Flybase genes) yielded significant clusters ($p<0.05$; none survived Benjamini correction) related to gland development, response to stimulus, embryonic development, metabolism, and cell growth (Tables 3 and 4). Several genes regulating neurophysiological processes were identified, including

pickpocket, *shot*, and *ebony*. Several genes involved in lipid metabolism were also identified, including *CG3812, CG9743,* and *CG6296* (Table 5).

Gene List Comparisons

Liang et al. (2012) identified 1219 genes that were differentially expressed in the brains of scouts and non-scouts. Only 11 of these genes were also found in the 142 behaviorally-regulated transcripts in our study, and this overlap was not significant (Hypergeometric Test; $p>0.05$). Alaux et al. (2009) identified 1396 genes that were differentially regulated in the brains of nurses and foragers. Only 25 of these genes were also found in our study (Table 6), but this is significantly more overlap than expected by chance (Hypergeometric Test; $p<0.01$). These genes included *cbt*, *insulin-like receptor*, *pinocchio*, and *Pbprp3*, an odorant binding protein. When directional expression changes were taken into consideration, there was a significantly greater overlap than expected by chance in genes whose expression was up-regulated in swarming versus non-swarming bees, and genes whose expression was up-regulated in nurse versus forager bees (Tables 7 and 8).

Discussion

We have identified a number of physiological and transcriptional differences between worker honey bees who depart with a swarm versus those who remain in the parental nest. Levels of *vitellogenin* were two to four times higher in swarming bees across both colonies that were examined, suggesting that physiologically, swarming workers are more nurse-like. In terms of patterns of gene expression in workers' brains, swarming behavior was associated with a change in the expression of only 142 genes, which is a relatively small number of genes compared to changes in expression across behavioral contexts for other scenarios—971 genes among virgin, mated, and laying queens (Kocher et al., 2008), 2607 genes between workers exposed to QMP versus a solvent control in a laboratory setting (Grozinger et al., 2003), and 1208-2670 genes between nurses and foragers (Cash et al., 2005; Whitfield et al., 2003). Despite a small number of genes being involved, there was a significant overlap with gene expression patterns associated with behavioral maturation. Furthermore, expression patterns in the brains of swarming bees were

more nurse-like, with significant overlap between genes up-regulated in swarming workers and genes up-regulated in nurses. Previous studies have found that the swarm is primarily made up of younger workers (Butler 1940; Gilley 1998). Although we did not know the ages of workers in our swarms, the swarming workers that we sampled had significantly higher levels of *vitellogenin*. *Vitellogenin* is an egg-yolk and storage protein (Wheeler and Kawooya 1990), a juvenile hormone regulator (Guidugli et al., 2005), and anti-oxidant protein (Seehuus et al., 2006), and can function in immunity (Amdam et al., 2004). *Vitellogenin* levels are highly correlated with behavioral maturation, with levels significantly higher in nurses than foragers (Nelson et al., 2007). Together with juvenile hormone, vitellogenin may also regulate the pace of behavioral maturation. Workers with suppressed *vitellogenin* levels begin foraging behavior earlier, and also have shorter lifespans (Nelson et al., 2007), suggesting that it has an important function in regulating social organization. The *vitellogenin* levels in our study suggest that workers are physiologically more nurse-like than they are forager-like. It also suggests that swarming workers, who are clearly more inclined to exit the hive and fly with a departing swarm than non-swarming workers, are motivated to leave the nest while still having high levels of *vitellogenin* that are characteristic of nurses, who are not attracted to light and tend to stay inside the nest as a consequence (Ben-Shahar et al., 2003).

We investigated whether there was any evidence of a difference in the reproductive potential of workers who stayed or left with swarms by looking at ovariole number, which predicts the likelihood of ovary activation in the absence of a queen (Makert et al., 2006). It has long been debated whether worker honey bees modify their behavior based on changing opportunities for direct reproduction (Breed et al., 1994). Workers typically do not reproduce, but under queenless conditions, some workers activate their ovaries and start laying unfertilized eggs which develop into male drones (Page and Robinson 1994; Miller and Ratnieks 2001). In a temporarily queenless situation, such as when a mother queen departs with a swarm and a replacement daughter queen has not yet taken over, such a reproductive opportunity is created for workers who stay if they activate their ovaries in the absence of the queen. Given the drop in relatedness that occurs for a worker between the siblings she reared when the old queen was present in the colony, and the nieces and nephews she will rear when the new queen takes over, a worker with high reproductive potential may choose to stay in the temporarily queenless colony to seize the reproductive opportunity that is created. That adult workers might take such an opportunity is supported by the observation that "rebel" larvae reared while colonies are temporarily queenless

during swarming have more activated ovaries with more ovarioles than workers reared in queenright colonies (Woyciechowski and Kuszewska 2012). However, we did not find that workers who stayed in colonies had more ovarioles than workers who left. This suggests that their decision to leave with the swarm is not motivated by the long delay in the production of new brood that could favor conditions necessary for ovary activation. Our results echo other observations that workers do not make choices to stay or go based on opportunities to increase their indirect fitness as relatedness asymmetries shift with the departure of a swarm and the takeover of a colony by a new queen (Rangel et al., 2009). Thus, an individual worker's decision to depart with the swarm or remain in the colony is likely regulated by other factors.

Whether a worker stays or goes with a swarm is clearly linked to changes in gene expression in their brains. Interestingly, these differences were not particularly large – only 142 genes were differentially expressed based on behavior, while 352 genes were differentially expressed between the two colonies and expression of 766 genes showed a behavior x colony interaction (meaning their expression patterns were differentially associated with behavior in the two colonies examined). There are many possible sources for variation between colonies, including genotype, presence of parasites/pathogens, and environmental conditions, and thus having a large colony effect is not surprising. However, it is somewhat surprising that only 142 genes were associated with the profound behavioral difference between swarming and non-swarming workers. Previous studies examining brain gene expression patterns in nurse versus forager bees found differences in expression of approximately 40% of the genes in the study (2670 genes; Whitfield et al., 2003). However, other studies examining brain gene expression differences in middle-aged, behaviorally distinct groups of bees found differences in a much smaller proportion of genes (1208 of 5563 transcripts tested; Cash et al., 2005). While there are significant differences in *vitellogenin* levels between swarming and non-swarming workers, other studies have demonstrated that all workers in a colony engorge themselves with honey before the swarm departs (Combs 1972), so it is possible that other physiological and metabolic parameters are relatively similar between swarming and non-swarming workers. Alternatively, levels of proteins, neurotransmitters, or neurohormones in the brain, or expression differences in the peripheral sensory organs, may be critical for triggering these differences in behavior. Finally, there could also be significant variation among swarming bees, such that these bees are not a uniform, consistent group, but rather represent different behavioral classes, such as bees specialized for building comb, storing food, caring for brood, etc. Thus, pooling across individuals may average

any underlying expression differences, similar to pooling across all workers in a hive. It is possible that analyzing large numbers of individuals would have allowed us to see separate classes of swarming and non-swarming bees.

Of the genes that were differentially expressed in swarming workers versus non-swarming workers, several were involved in processes associated with behavioral maturation. Nurse bees have significantly larger hypopharyngeal glands (Crailsheim and Stolberg 1989), which are used to produce brood food, and one of the major GO categories was "gland development". In combination with high levels of *vitellogenin,* this suggests that swarming workers are in a nurse-like state. Indeed, previous studies of swarming workers found they had larger hypopharyngeal glands (Zeng et al., 2005). There were also differences in genes involved in lipid metabolism (*CG3812, CG9743, CG6296, CG6718,* and *Pi3K92E*). Differences in lipid levels between swarming and non-swarming bees have not been shown, but nurse bees have significantly larger lipid stores than forager bees (Panzenbock and Crailsheim 1997), and loss of these lipid stores can accelerate behavioral maturation (Toth and Robinson 2005; Toth et al., 2005). Several genes involved in sensory perception were also differentially expressed. These genes included vision genes such as *cinnabar* (*cn*), which is involved in ommochrome biosynthesis (Beadle and Ephrussi 1937); *charlatan* (*chn*), which is involved in eye development and sensory organ development (Escudero et al., 2005); and *CG33174*, which is involved in phototransduction (Leung et al., 2008). Several olfactory genes were also identified, including *pigeon*, which is involved in olfactory and associative learning; *pinocchio* (*smi21F*), which is involved in olfactory behavior (Rollmann et al., 2005); and *Or83a*, which is involved in olfactory receptor activity and sensory perception of smell (Störtkuhl and Fiala 2011). Finally, genes involved in neurophysiology were differentially expressed, including *pickpocket*, which is involved in sodium channel activity and sodium ion transport (Zhong et al., 2010); *shot*, which is involved in protein binding and mushroom body/neuron development (Applewhite et al., 2010); and *ebony*, which is involved in beta-alanyl-dopamine synthase activity. These sensory perception genes could be important for helping workers identify the presence or absence of the queen while the swarm is airborne.

Comparisons with previous studies demonstrated that there was a significant correlation between the genes associated with swarming behavior and aging. Indeed, of the overlapping genes, there was a tendency for genes up-regulated in nurse bees to be up-regulated in swarming bees (overlap

of12 genes, $p<0.037$; Table 5). However, there was no significant overlap with genes that were differentially expressed between scouting bees and non-scouting foragers, suggesting that genes associated with "novelty seeking" are highly specialized and not generally associated with the genes (and behaviors) that are expressed by workers when they pour out of their natal nest and take to the air for the first leg of the swarming process.

These results suggest that in addition to a division of labor based on behavioral maturation in established colonies, worker honey bees also have a division of labor during swarming, during which a distinct group of workers leaves the colony with the old queen to establish a new colony at a different location. These swarming bees appear to be generally more nurse-like than the bees that remain in the colony, in terms of levels of *vitellogenin* and gene expression. However, overall the differences in brain expression were not very large, suggesting that other factors, perhaps increased sensitivity to swarming cues due to changes in expression patterns in sensory organs or altered levels of circulating neuromodulators, may trigger the decision to leave during the swarming process.

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Figures

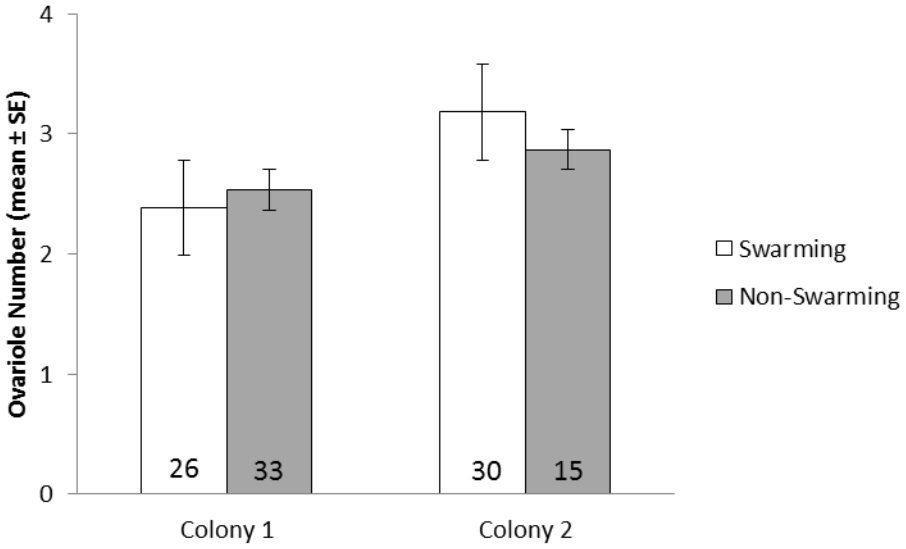

Figure 3-1. Ovariole number of swarming and non-swarming worker bees. There is no significant difference in ovariole number between swarming and non-swarming workers. t-test; Colony 1: t(51)=-0.5690, p=0.2860; Colony 2: t(35)=0.9710, p=0.1691. The total number of individuals assayed in each sample group is denoted in the bottom of the bar.

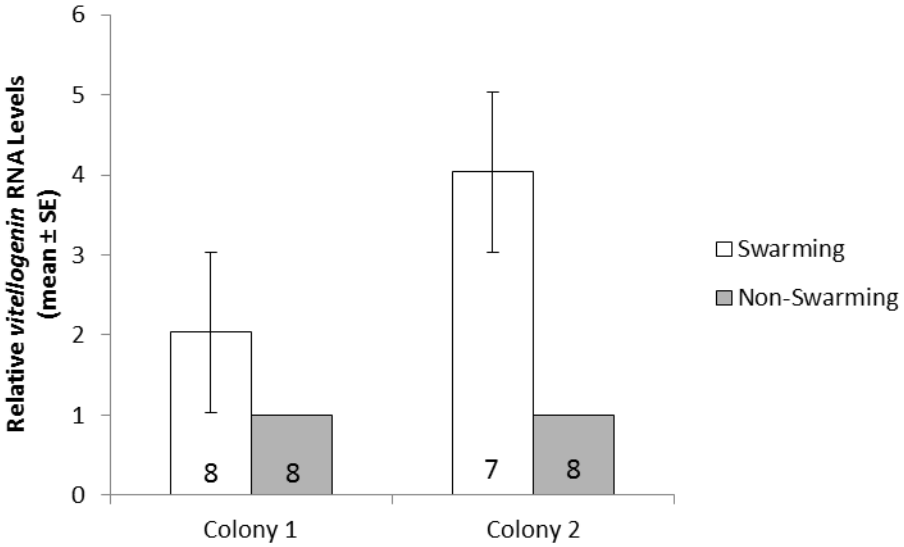

Figure 3-2. Relative *vitellogenin* RNA levels. qRT-PCR analysis was performed as described in the methods. *Vitellogenin* RNA levels were significantly higher in swarming versus non-swarming workers: two-factor ANOVA; behavior: $F(1,24)=25.6626$, $p<0.001$; colony: $F(1,24)=1.2403$, $p=0.2764$; behavior x colony: $F(1,24)=3.7591$, $p=0.0644$). The total number of replicates used in each sample group is denoted in the bottom of the bar.

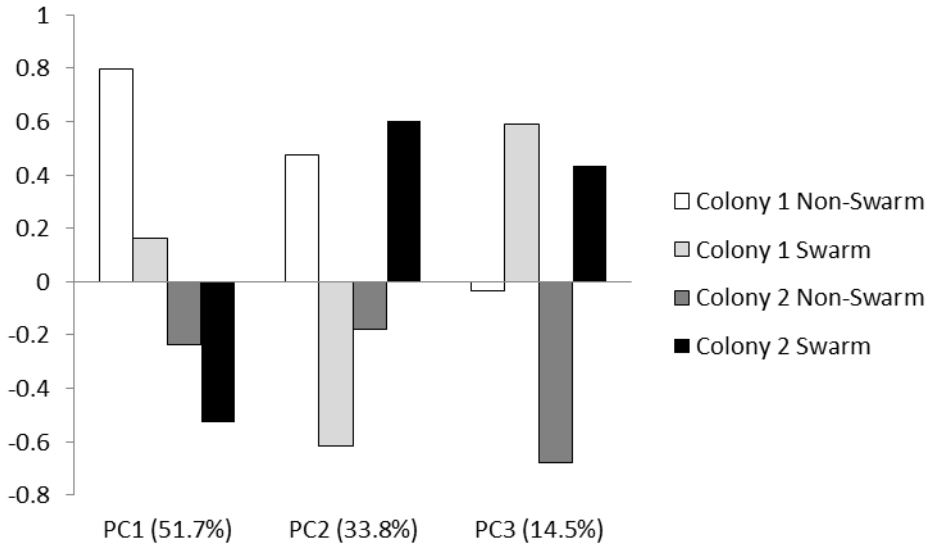

Figure 3-3. Principle components analysis. A principle components analysis was performed to examine underlying patterns of gene expression among the 142 behavior-effect genes. PC1 (Colony): 51.7%; PC2 (Behavior x Colony): 33.8%; PC3 (Behavior): 14.5%.

Table 3-1. Primers for qRT-PCR of *vitellogenin* RNA levels.

Gene	Forward	Reverse	Reference
Actin	CCTAGCACCATCCACCATGAA	GAAGCAAGAATTGACCCACCAA	Grozinger et al., 2003
eIF-S8	TGAGTGTCTGCTATGGATTGCAA	TCGCGGCTCGTGGTAAA	Richard et al., 2007
Vitellogenin	TTGACCAAGACAAGCGGAACT	AAGGTTCGAATTAACGATGAAAGC	Fischer and Grozinger 2008

Table 3-2. Numbers of differentially expressed transcripts between swarming and non-swarming workers.

	Number of Transcripts		Number of Unique Transcripts		
	Total	Total Unique	Colony	Behavior	Colony x Behavior
FDR<0.05	4,466	2,035	797	403	1,855
FDR<0.01	1,808	859	352	142	766

Table 3-3. Gene ontology analysis of the 142 behavior-effect transcripts.

Cluster	GO Term	GO Biological Process	p-value	# Genes
1	GO:0048732	gland development	0.0045	7
	GO:0022612	gland morphogenesis	0.019	5
	GO:0007435	salivary gland morphogenesis	0.019	5
	GO:0035272	exocrine system development	0.041	5
	GO:0007431	salivary gland development	0.041	5
2	GO:0050896	response to stimulus	0.021	16
3	GO:0007390	germ-band shortening	0.0013	4
	GO:0009790	embryonic development	0.046	10
4	GO:0006629	lipid metabolic process	0.034	7
5	GO:0001558	regulation of cell growth	0.046	3
6	GO:0009790	embryonic development	0.046	10

Table 3-4. Flybase gene IDs from gene ontology analysis clusters.

Gland Development	Response to Stimulus	Embryonic Development	Lipid Metabolic Process	Regulation of Cell Growth	Embryonic Development
FBgn0013770	FBgn0000473	FBgn0015279	FBgn0053174	FBgn0015279	FBgn0015279
FBgn0034539	FBgn0034539	FBgn0013984	FBgn0034997	FBgn0013984	FBgn0013984
FBgn0015279	FBgn0015279	FBgn0043364	FBgn0030421	FBgn0013733	FBgn0043364
FBgn0043364	FBgn0013984	FBgn0015371	FBgn0015279		FBgn0015371
FBgn0004618	FBgn0037322	FBgn0001168	FBgn0039470		FBgn0001168
FBgn0001168	FBgn0016926	FBgn0011296	FBgn0036053		FBgn0011296
FBgn0003720	FBgn0010309	FBgn0004567	FBgn0039756		FBgn0004567
	FBgn0014033	FBgn0003499			FBgn0003499
	FBgn0043364	FBgn0003720			FBgn0003720
	FBgn0000527	FBgn0003882			FBgn0003882
	FBgn0004618				
	FBgn0001168				
	FBgn0011296				
	FBgn0013548				
	FBgn0004242				
	FBgn0003882				

Table 3-5. Annotation of a selection of significantly, differentially regulated genes in swarming versus non-swarming workers.

Annotation Cluster	Name	Flybase ID	Gene Name	Description	Associated Reference
Neurophysiology	AM03105	FBgn0065108	pickpocket (ppk16)	sodium channel activity; sodium ion transport	Zhong et al., 2010
	AM04215	FBgn0013733	short stop (shot)	protein binding; mushroom body/neuron development	Applewhite et al., 2010
	AM12488	FBgn0000527	ebony (e)	beta-alanyl-dopamine synthase activity	
Lipid Metabolism	AM03302	FBgn0030421	CG3812	phospholipid biosynthetic process	Kuhnlein 2012
	AM09781	FBgn0039756	CG9743	oxidation-reduction process	
	AM12065	FBgn0039470	CG6296	triglyceride lipase activity	
	AM12373	FBgn0036053	CG6718	calcium-independent phospholipase A2 activity	
	AM12186	FBgn0015279	Pi3K92E	lipid kinase activity; phospholipid metabolic process	Witte et al., 2009
Sensory Perception-Vision	AM06501	FBgn0000337	cinnabar (cn)	ommochrome biosynthetic process; ocelli pigments	Beadle and Ephussi 1937
	AM09788	FBgn0015371	charlatan (chn)	eye development; sensory organ development	Escudero et al., 2005
	AM05375	FBgn0053174	CG33174	lipoprotein lipase activity; phototransduction	Leung et al., 2008
Sensory Perception-Olfaction	AM11479	FBgn0010309	pigeon	olfactory learning; associative learning	
	AM11549	FBgn0016926	pinocchio (smi21F)	olfactory behavior	Rollmann et al., 2005
	AM00253	FBgn0037322	Or83a	olfactory receptor activity; sensory perception of smell	Störtkuhl and Fiala 2011

Table 3-6. List of genes overlapping between swarming/non-swarming workers and nurses/foragers.

Name	Flybase ID	Gene Name	Brief Annotation
AM00378			
AM00379			
AM00446			
AM00964			
AM01183			
AM01689			
AM02502	FBgn0040900	CG17777	
AM02742	FBgn0043364	cbt	development; autophagic cell death
AM02757	FBgn0032192	CG5731	enzyme; carbohydrate metabolism
AM05754	FBgn0039075	CG4393	
AM05952	FBgn0036121	CG6310	
AM06766			
AM07226	FBgn0025625	CG4290	salt inducible kinase; response to starvation
AM07697	FBgn0034539	CG11159	autophagic cell death
AM08121	FBgn0031327	CG5397	carboxyl esterase
AM08479	FBgn0013984	InR	insulin-like receptor
AM09209	FBgn0039728	CG7896	
AM09471	FBgn0011281	Pbprp3	odorant binding protein
AM09781	FBgn0039756	CG9743	fatty acid desaturase
AM10423	FBgn0037977	Ect3	galatosidase activity; autophagic cell death
AM10643			
AM10769			
AM11549	FBgn0016926	smi21F	(pinocchio) olfactory behavior
AM12193	FBgn0031692	TpnC25D	calcium ion binding
AM12575			

Table 3-7. Directional overlap of swarming/non-swarming and nurse/forager gene lists.

Gene List	Up-regulated in swarming workers (99 transcripts)	Representation Factor and p-value	Up-regulated in non-swarming workers (43 transcripts)	Representation Factor and p-value
Up-regulated in foragers (513 transcripts)	7	1.8; p<0.099	2	1.2; p<0.486
Up-regulated in nurses (883 transcripts)	12	1.8; p<0.037	4	1.4; p<0.339

Table 3-8. List of genes overlapping between swarming workers and nurses.

Name	Flybase ID	Gene Name
AM00378		
AM00379		
AM02502	FBgn0040900	CG17777
AM05952	FBgn0036121	CG6310
AM07697	FBgn0034539	CG11159
AM08121	FBgn0031327	CG5397
AM09209	FBgn0039728	CG7896
AM09781	FBgn0039756	CG9743
AM10423	FBgn0037977	Ect3
AM10643		
AM10769		
AM12575		

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Chapter 4

Conclusions and Future Directions

Honey bees provide an excellent model system in which to examine the communication systems underlying complex social behavior. Swarming behavior is a fascinating example of social communication where the majority of the workers leave the hive with the old queen to found a new nest in a new location, while the remaining workers are left in the original colony with developing queens. Until now, the chemical communication systems regulating this behavior, as well as the neurophysiological and molecular mechanisms underlying worker response to these signals and/or cues, had not been characterized.

In Chapter 2, we demonstrated that queens at swarm liftoff tend to produce greater quantities and significantly greater numbers of volatile compounds than before swarming. Also, workers from swarms were significantly more attracted to swarming queens than non-swarming queens. These changes in queen volatiles at liftoff suggest that queens are modifying their chemical signals during the swarming process, and these results suggest that queens play a more active role in the swarming process than the passive role that has been previously described (Simpson 1958).

While we have begun to answer some of the questions regarding the role the queen may be playing in the swarming process, a number of questions remain to be answered. To demonstrate that these are pheromones, it will be necessary to identify the remaining 12 candidate compounds that were identified from the SPME samples, obtain purified individual compounds (either through commercially or through chemical synthesis), and then test them individually and in combination on swarms. These "swarm pheromones" may simply attract swarming workers to the queen, or may actually trigger swarm liftoff: different behavioral assays would need to be developed to test both behavioral responses (see below for further discussion). Since there was no single compound present in all liftoff queens, or compounds consistently found only in liftoff queens, it does not appear that there is a distinct "swarming pheromone" produced by the queen that induces the swarming behavior or bivouac liftoff, but rather that blends of chemicals are involved. Furthermore, identifying specific chemical compounds or blends that will interrupt the swarming process and prevent bees from leaving the colony could be of particular interest to

beekeepers, who try to minimize the occurrence of swarms as the process disrupts hive productivity and honey production.

Further studies are also necessary to understand the mechanisms underlying the production of these altered chemical blends during swarm liftoff. It is possible that the queen releases different blends of pheromones in order to stimulate the workers to begin certain preparations for swarming. If so, it remains to be seen which factors trigger this novel chemical blend to be released, and how the queen knows to initiate this chemical release. Or, on the other hand, the queen's change in chemical profile could be in response to the workers' actions as they initiate the swarming process.

Since swarming behavior is initiated well before liftoff, it is possible that there are other pheromones, aside from reduced transmission of queen mandibular pheromone (QMP), that trigger this process. If so, these pheromones could potentially be identified by collecting chemical samples (volatile or gland-produced) from queens at different stages of the in-hive swarming process (queen rearing, worker engorgement, decreased oviposition, etc.).

We also do not know if this chemical blend serves simply to confirm the presence of the queen, or if it is actively altering the behavior of the swarm, such as triggering liftoff. It is already known that 9-ODA, a component of queen mandibular pheromone (QMP), helps attract workers to and stabilize the swarm cluster (Butler and Simpson 1967) and that workers will return to the colony if the queen does not join them at liftoff (Morse 1963, Simpson 1963). Previous studies have only focused on QMP components' effects, but we have identified a number of new compounds for testing that may also play a role in worker attraction to the swarm or in advertising the queen's presence to the swarm.

In Chapter 3, we examined anatomical, physiological, and transcriptional differences in worker bees that depart with the swarm versus those left in the hive. We found no significant difference in reproductive potential (number of ovarioles), but levels of vitellogenin were significantly higher in swarming bees than non-swarming bees, suggesting that swarming workers are physiologically more nurse-like. Swarming behavior was also associated with a small number of gene expression differences, but these significantly overlapped with gene expression patterns

associated with behavioral maturation; expression patterns in the brains of swarming bees were more nurse-like.

The profound behavioral distinction between swarming and non-swarming workers suggests that these two groups of bees are differentially responsive to different sensory cues. Future studies could examine these cues in more detail. For example, swarming workers could be more phototactic (which is typically a forager trait), which could aid in the flight process during swarming, or in swarm orientation. They could also be more sensitive to swarm-specific chemical signals or cues. This would allow swarming workers to rapidly identify the chemical cues signaling the queen's presence while the swarm is in flight, a crucial part of the swarming process since a swarm will fail if the queen does not accompany them to the new nest site. Importantly, using electroantennograms coupled to gas chromatography to compare the sensitivity of swarming and non-swarming workers to the different volatile chemicals produced by swarming queens during liftoff could rapidly identify specific chemicals that play important roles in the swarming process.

We are also interested in further examining the theory that swarming behavior originated as a migratory behavior in response to unfavorable conditions (Ribbands 1964). If this is true, there could be interesting behavioral, physiological, and molecular parallels between swarming and absconding workers. A chemo-genomics study, similar to the one we have conducted here using swarming and non-swarming workers, could elucidate some of these similarities.

While these studies have shed new insights into the chemical, physiological, and genomic factors underlying swarming behavior in honey bees, it is clear that there are many new directions to be explored. We have only begun to identify and characterize the queen-produced chemical blends involved in the swarming process, and how workers respond to these chemical cues. Our results demonstrate that swarming and non-swarming workers represent distinct physiological and behavioral states, and they are likely differentially responsive to these chemical cues, but additional studies could further elucidate the differences between these two groups.

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