Behavioral and physiological changes in honey bee (*Apis mellifera*) queens during swarming events

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Abstract

Within a large and growing honey bee colony, overpopulation results in the initiation of a reproductive process known as swarming, which divides one strong colony into two separate and smaller colonies. When a colony swarms, roughly two-thirds of its worker population departs with the original queen to establish a new nest. Workers are aware of the queen’s presence in the airborne swarm via the pheromones that she emits. Our previous work has indicated that a queen’s pheromone production increases prior to liftoff. To determine how queens prepared for liftoff and how worker-produced signals facilitate this process, swarming was induced in colonies and queens were monitored through the process for pheromone production and changes in body temperature and activity level. These queen metrics were related to simultaneous activities of workers in the swarm. The thoracic temperatures of queens rose as swarms prepared for liftoff, and workers selectively heated the area around the queen throughout the process. There were also strong links between increases in temperatures as swarms prepared for liftoff and total pheromone emissions and the number of chemical components present in the pheromones that queens produced. These changes in the physiology and behavior of queens during the swarming process suggest that queens interact with bivouacking workers in a way that facilitates her ability to advertise her presence in the swarm when it lifts off for flight. Future studies will focus on isolating the queen from these sources of information and observing whether this prevents her from preparing for swarm departure toward a new nest site.
**Introduction**

**Apis mellifera: a complex insect society**

In the modern era, biologists have exerted great effort to explore miniature insect societies that are hidden in plain sight. One species that has received widespread scientific consideration is *Apis mellifera*, the European honey bee. The native range of this species of bee extends from the northern extreme of Europe down through the southern tip of Africa. Although not native to the Americas or Australia, *A. mellifera* was brought across the ocean and introduced to these continents where it now reigns as the most widespread pollinator of commercial crops (Winston, 1987). Honey bees, in conjunction with other insect pollinators, are used to pollinate 35% of the world’s crop supply on an annual basis (Klein et al., 2007), so the importance of understanding their complex societies extends beyond simple scientific curiosity, it is critical for understanding how we can meet the food demands of a growing human population.

**Colony organization and reproductive division of labor**

A honey bee society, known as a colony, is organized into three distinct castes, each of which has a separate function. The single most important member of the colony is the queen, who is solely responsible for carrying out all egg-laying responsibilities within the colony. Upon emerging from her cell, the virgin queen undergoes a number of mating flights during the first week of her life. Over the course of these flights, she will mate with an average of 12 male drones (Tarpy et al., 2004). The sperm received from these drones fertilizes all of the eggs that...
she will lay over the course of her lifetime. She will spend most of the rest of her life in the colony laying eggs, barring a colony fission event where she will depart with a portion of the colony’s population to help establish a new nest (Winston, 1987).

The queen produces all of the offspring in her colony, giving rise to two other castes of related individuals whose sex depends on their number of chromosomes. Honey bees exhibit a haplo-diploid sex determination system wherein a female worker possesses two sets of chromosomes, one of which is inherited from her mother and the other from her father. Males, on the other hand, are haploid and inherit only a single set of chromosomes from their mother (Mackensen, 1951). Because honey bee queens mate multiply, each queen gives rise to a colony of workers that are half-sisters, all of whom share the same mother but have different fathers, while all drones share only the same mother (Hamilton, 1964). The vast majority of the queen’s progeny are members of the female worker caste, who perform tasks related to regular colony upkeep.

Honey bee societies exhibit behavioral polytheism, wherein workers vary the tasks that they perform over the course of their life depending on their age. Responsibilities early in life are limited to within-colony tasks, such as caring for brood, feeding developing larvae, attending the queen, building comb, and receiving food from foragers. Later in life, workers graduate to more dangerous tasks outside the nest, such as guarding the nest’s entrance and foraging (Seeley, 1982). The primary function of the drone caste is to find a queen with whom to mate. Their physiology is particularly well-suited to this task; they have wings that are much larger than those of workers and are designed for sustained flight (Lensky et al., 1985). They
also have eyes that cover most of their head’s surface so that they can easily spot a queen in flight (Dade, 1977). A drone does not assist in regular hive upkeep and dies after mating with the queen and depositing his semen into her (Winston, 1987).

With this complex organization of their societies, honey bees meet the criteria for eusociality as defined by E.O. Wilson in 1975: “(1) individuals of the same species cooperate in caring for the young; (2) there is a reproductive division of labor, with more or less sterile individuals working on behalf of fecund nest mates; (3) and there is an overlap of at least two generations in life stages capable of contributing to colony labor, so that offspring assist parents during some period of their life” (p. 398). The average colony therefore consists of its reproductive individuals (the single queen and up to a thousand male drones who dedicate their lives to this task) and the tens of thousands of female workers who take care of all other tasks within the colony and beyond (Winston, 1987).

**Communication within honey bee societies through dancing and pheromones**

Within social insect societies, there has been widespread documentation of communication between members to signal an alarm against attackers, to successfully navigate back to the nest, or to locate mates. Modes of communication in honey bees are particularly striking amongst social insect species. One form of signaling employed by honey bees is the emission of chemicals known as pheromones (Winston, 1987). Karlson and Luscher (1959) define pheromones as chemicals that are emitted by an organism that trigger an innate behavior in other members of the same species.
Honey bees produce distinct pheromones for a plethora of tasks. One well-documented pheromone used within honey bee colonies is the secretion by workers of a recruitment pheromone from their Nasanov glands. This pheromone is released by raising the abdomen, exposing the gland between abdominal tergites, and then rapidly fanning the wings to disperse the chemical throughout the ambient environment. This blend of chemicals is an attractant to other workers and is often released near the colony entrance to help foraging workers successfully orient to their nest (Winston, 1987). Examples of other pheromone interactions within honey bee societies include chemical suppression of ovarian development in workers by the presence of queen-produced pheromones (Butler, 1959) and the release of alarm pheromone to alert nest members to a perceived threat to the colony (Free and Simpson, 1968).

Pheromone production by the queen is an important mechanism by which she can signal her presence to the workers within her colony. This signaling is of the utmost importance within the colony because without an egg-laying queen, the colony has no means of maintaining the growth of its worker population. The most studied components of queen pheromones include 9-oxodecenoic acid (9-ODA) (Callow and Johnson, 1960; Barbier and Lederer, 1960) and 9-hydroxy-(E)-2-decenoic acid (9-HDA), both of which are released from the queen’s mandibular gland, which is located within her head (Callow, Chapman and Paton 1964; Butler and Fairey 1964). These chemicals, in concert with three other mandibular secretions, are known as queen mandibular pheromone (QMP) and together they regulate reproduction within the colony. 9-ODA is the component that is present in greatest abundance, followed by the two chiral forms of 9-HDA, with lesser amounts of two remaining aromatic compounds.
(methyl p-hydroxybenzoate and 4-hydroxy-3-methoxyphenylethanol) in the blend. All five components are necessary for full potency and removal of any one component has been shown to reduce the effect of the remaining chemicals by up to 50% (Winston and Slessor, 1992). When these pheromones circulate within the colonies in high quantities, it informs workers that there is a healthy, egg-laying queen present. Declining levels of QMP signal to workers that the queen’s health is failing or that she is absent entirely. Under these conditions, workers will begin rearing a replacement queen (Butler, 1961). In addition to signaling the queen’s presence to the workers, these pheromones also attract male drones to queens during their mating flights (Gary, 1962; Butler and Fairey, 1964).

Beyond their use of pheromones for communication, honey bees also exchange information with nest mates using their well-characterized waggle dance (fig. 1). Returning foragers waggle dance in order to relay the location of a suitable source of pollen or nectar to other workers so that naïve workers may also visit that site and bring food back to the colony. Foragers most often begin this dance on the comb near the entrance. The dance is a series of figure eight motions that tells the other workers the direction and distance to the food source (fig. 1). During a typical dance, a forager will proceed in a straight line run for a short period of time, vigorously shaking her abdomen side to side and emitting brief buzzing noises. Her run is followed by an arcing return in either direction (left or right) that brings the dancer back to the starting point of the run, where she will repeat the entire dance once more (fig. 1). The number of “waggles” during the straight run and the tempo of the dance (von Frisch, 1967), in combination with how a dancer vibrates the comb (Nieh and Tautz, 2000) provide information on distance to the food source. The direction of the straight run creates an angle relative to the
vertical that is translated relative to the sun’s position in the sky; individuals going to forage use this information to navigate in the proper direction upon their exit from the hive (von Frisch, 1967). As the foragers dance to communicate the location of a food source, they are surrounded by a crowd of attentive workers who “read” the dance for its pertinent information. In the darkness of the colony, workers follow the dance through physical contact and by feeling vibrations on the comb. This same dance is used to communicate potential sites for establishing a new nest during the colony fission process (see below; Winston, 1987).

Figure 1. Honey bee foragers use the waggle dance to communicate the location of food sources to workers within the colony. The waggle dance follows a figure eight pattern where foragers proceed a short distance in a straight line while vigorously shaking their abdomen side to side to indicate distance of the food source from the colony before looping back to repeat their circuit. The orientation of the straight run in relation to gravity informs naïve foragers which direction to head (in relation to the sun’s position) upon exit from the hive. Figure obtained from Winston, 1987.
Swarming as a method of reproduction on a colony scale

While individual-level reproduction is achieved within a colony through drone mating and the laying of eggs by the queen, a second form of reproduction that involves queens is called swarming, and it occurs at the colony level. During this group reproductive process, the majority of workers and their mature queen will depart from the original colony in search of a nest site to start a new colony. This process most often occurs in late spring during the months of May and June, when a colony has had a chance to recover resources and population numbers from winter lows (Burgett and Morse, 1974).

The mechanism by which workers time the initiation of the swarming process is thought to be based in the pheromone signals that they receive from the queen. The queen advertises her continued presence to the colony via secretion of QMP, a pheromone that is known to suppress queen rearing by workers. QMP is distributed via direct antennal contact between the queen and her retinue workers who attend her as she moves through the colony. These retinue workers then distribute QMP throughout the colony as they interact with other workers (Juska et al., 1981; Seeley, 1979; Winston and Slessor, 1992). When QMP levels are high enough, workers perceive that they have a healthy queen in the colony, but when levels fall, it signals to them that they need to start rearing a new queen (Winston, 1987). Dropping levels can indicate to workers that: 1) their colony has become queenless or 2) their colony is crowded enough that the swarming process can begin. These responses suggest that there is a certain minimum threshold of QMP that must be maintained within a colony that correlates with the relative size of the community. If QMP levels fall below this threshold, it triggers workers to rear a new
queen, which is the first step in a swarming event. In larger colonies, the transmission of QMP throughout the colony by contact between workers is slowed under congested conditions. This allows the worker population to start rearing a new crop of queens despite the mature queen’s continued presence in the colony (Winston and Slessor, 1992). A study by Winston and Slessor (1992) found that a swarming event in a crowded colony could be delayed by up to 25 days by providing colonies with supplementary supplies of QMP.

Preparation for a swarming event usually begins approximately 2 to 4 weeks prior to swarm departure. During this phase, the primary focus of the workers is rearing new queens. Because queens are responsible for laying the eggs to keep worker populations strong, steps are taken to ensure that a line of succession is in place (i.e., the rearing of a new queen) when a swarm departs with the old queen. As the first step in this process, workers construct wax queen cups on the edges or bottom of honey comb sections where new queens are reared. Queens will either lay eggs directly into these cups, or workers fill them by moving young larvae to these cups from other brood cells elsewhere in the colony. It is important to note that upon hatching from the egg, all larval females have the potential to mature into queens. The only factor that determines whether a female larva will mature into a queen or a worker depends on the type of food that it is fed during this development phase. As the eggs placed in queen cups hatch into larvae, workers feed them a diet that consists of a special food source called royal jelly that spurs their development into queens. Royal jelly is distinguished from the food that is provided to other larvae by a higher concentration of mandibular gland secretions (Jung-Hoffman, 1967) and the presence of the royalactin protein, which shortens the larval period of development and increases both ovarian development and body size (Kamakura, 2011). As
larvae continue to grow, workers build up and elongate the queen cells, eventually sealing them off to allow for larvae to pupate and later emerge as adult queens (Butler, 1957). Workers will seal an average of 15-25 queen cells immediately prior to and after a swarm departs from its original nest, with departure usually falling within a day or two after the first queen cell is sealed (Winston, 1987).

In addition to rearing new queens, workers also undergo other preparations prior to the departure of a swarm. In the weeks leading up to a swarming event, workers will increase their feeding of the queen to spur higher rates of egg laying in an effort to maximize the amount of sealed brood (pupating larvae) that will be in the colony at swarm departure. This final crop of offspring from the mature queen will ultimately replenish the colony’s work force after the sudden population drop that occurs when the majority of the bees leave with the swarm (Winston, 1987). Approximately one week before departure, this trend is rapidly reversed and workers reduce the frequency with which they feed the queen. As a fully mature and egg-laying queen is too heavy to fly, this reduction in feeding and concurrent shrinking of her ovaries will make her light enough to be able to fly when the swarm departs (Taranov and Ivanova, 1946; Allen, 1955, 1956, 1960). Beginning approximately 10 days before swarming, workers consume copious amounts of honey to fill their stomachs in preparation for departure. While non-swarming workers normally carry an average of 10mg of honey in their stomach, workers who will swarm engorge themselves on an average of 36mg of honey per bee (Combs, 1972). When the swarm departs, the food reserve that is carried by the workers will be all of the sustenance that is available to them until a new nest is founded and workers begin foraging once more (Winston, 1987).
On the day that the swarm leaves from the nest, the behavior of workers within the colony undergoes a radical shift. A few hours before swarm liftoff, rates of buzz running increase as workers quickly scamper across the comb, vibrating their wings as they go to excite the worker population within the colony for an impending exodus. The queen is also antagonized in a similar manner; she is subjected to chasing and biting by workers as they prepare for the swarm departure. Ultimately, the workers will exit the nest in a torrential outpouring from the colony as the queen and 60% of the colony’s population begin their journey toward founding a new nest site (Winston, 1987). The largest portion of the departing bees will be younger in age, with up to 70% of bees travelling with the swarm being less than 10 days of age (the average lifespan of a summer bee is ~30 days). The importance of the younger bees departing with the swarm lies in the fact that after colonization of a new nest site, it will take up to 21 days until the queen’s first batch of offspring emerge to rebuild the swarming workers’ population numbers (Butler, 1940). The remaining members of the original colony will utilize existing colony resources to rear the departed queen’s final batch of worker brood and her developing crop of daughter queens (Winston, 1987).

The first of the virgin queens will usually emerge approximately one week after the first (or prime) swarm issues from the parental nest (Otis, 1980). A few days after her emergence, the unmated queen will leave the colony with a subsequent afterswarm if the colony’s population is large enough (Winston, 1979; Otis, 1980). An average of one or two afterswarms will depart the colony after the first swarm, each with a newly emerged virgin queen. In a study by Otis (1980), the average size swarms issuing sequentially from a single colony were 16,000 workers for the first swarm, followed by 11,500 for the second, and 4,000 for the third. A
colony will issue as many afterswarms as it can, as long as there is a sufficient worker population within the original colony, with a strong positive correlation observed between the number of afterswarms and the amount of sealed brood present at the time of the primary swarming event (Winston, 1987).

The presence of an emerged virgin queen is usually enough to prevent other queens from emerging from their cells, with workers and the new queen working together to destroy the remaining queen cells and kill their occupants if no more afterswarms will issue from the colony (Caron and Greve, 1979). In an instance where more than one virgin queen emerges simultaneously and no further afterswarms are imminent, the two queens will seek out one another within the colony and fight. The queen that sustains more injury than the other will often be balled and killed by workers (Robinson, 1984). During balling, workers will cluster tightly around the queen and vibrate their fight muscles to increase the temperature within the cluster to lethal temperatures. With a new queen at the helm of the colony, she will begin mating flights and take over egg-laying responsibilities within the colony. In this way, strong colonies are able to divide into two or more fission colonies (Winston, 1987).

It is important to note that when a swarm departs from the colony, its members have not selected a location for constructing their new nest. Instead, the cloud of departing bees will fly a short distance from the hive and land together on a tree branch or another suitable surface, clustering around the queen in what is known as a bivouac (fig. 2). Recruitment pheromones are used to help the airborne swarm orient to the bivouac as it forms (Morse and Boch, 1971; Ferguson et al., 1979; Free et al, 1981). Initial orientation for landing is achieved
visually, with honey bees landing and quickly beginning to scent-fan with their Nasonov glands to recruit both the queen and other workers to the bivouac’s location (Morse and Boch, 1971; Ferguson et al., 1979; Free et al, 1981). The presence of the queen further assists in attracting the airborne bees. The active secretions of her mandibular gland have a two-fold function within the swarm. While airborne 9-ODA attracts airborne workers, 9-HDA plays a critical role in stabilizing the swarm by preventing its premature dissolution prior to the selection of a new nest site (Morse, 1963; Simpson and Ridel, 1963; Butler et al, 1964; Butler and Simpson, 1967; Morse and Boch, 1971; Avitable et al., 1975).

It is here within the bivouac that the vast majority of bees will remain in a quiescent state while a small proportion of the workers (~3-5% of the overall swarm) serve as scouts who will search for a suitable nest site (Gilley, 1998; Seeley and Buhrman, 1999). Scouts departing from the bivouac scour the surrounding area for natural or man-made cavities, inspecting them, and communicating through waggle dances the location of suitable sites to the workers back in the bivouac (Winston, 1987). Using a complex decision-making process, the swarm will ultimately decide upon a single nest site over a period of time that ranges from a few days to a week. After an excitation process wherein scouts rouse the quiescent worker population, the bivouac dissolves and travels to its new nest site to begin the process of creating a new colony (fig. 2; Seeley and Visscher, 2004).
In their review of group decision making in nest-site selection, Seeley and Visscher (2004) present three criteria that a honey bee swarm must fulfill in the process of choosing a new home. They must achieve accuracy in selecting a site that sufficiently meets the need of the colony, which include adequate space for colony resources and brood rearing. The selected location must also provide protection against the elements and other non-nest mate organisms such as predators or competing honey bee robbers that would seek entry into the hive. There is also emphasis placed on a speedy decision, hastened by the swarm’s precarious exposure to the elements and its limited food cache (the honey that the workers carried with them in their stomachs upon departure from the original colony). The final critical element in selecting a nest site is making a unified decision. Because the queen is responsible for all egg laying, a swarmis
dependent on their queen to rebuild the worker population once the new colony is founded, so it is critical that she travels with the workers throughout the entire process. Research has focused on deciphering the mechanism by which a honey bee swarm is able to fulfill these three criteria in their selection of a new home (Seeley and Visscher, 2004).

The process of nest-site selection begins with scouts departing from the swarm in search of potential locations. After finishing her survey of a potential nest site, a scout will return to the bivouac and communicate its location via a waggle dance. With many scouts out searching, 10 or more sites, located up to a few kilometers away from the bivouac location, may fall under consideration (Winston, 1987). Seeley and Buhrman (1999) demonstrated that, during the initial search phase, dancing for the different sites is relatively balanced, with no single site dominating the proceedings. Over the course of a few days, however, one site eventually gains momentum, overwhelming the others until it is the only site that is advertised. Usually within an hour of reaching the point of unanimous dancing for a single location, the bivouac will dissolve and fly to the new nest site.

At the scale of individual dancers, scouts tend to dance for a period of time and then their activity winds down until they are no longer advocating for a particular location via dancing. While some dancing scouts will switch their support to another site, the primary mechanism by which a quorum is achieved is by having scouts who are dancing for non-selected locations cease dancing for that particular location over time. Thus, for a particular site to emerge as the winner, it requires other scouts to agree with that choice as the decision-making process proceeds. This phenomenon suggests that there exists a built-in mechanism by which
scouts lose interest in dancing or participating in the decision-making process, passing the task over to the next “generation” of scouts and dancers (Seeley and Buhrman, 1999). Ultimately, through a sharing of this complex task, many bees partake in the evaluation of multiple potential nest sites to eventually rule out alternatives and agree upon a single location that will meet the needs of the swarm (Seeley and Visscher, 2004).

Complex criteria determine what constitutes a high quality nest site to a honey bee (Franks and Dornhaus, 2003). Upon discovery of a site, scouts spend a large amount of time examining it by crawling along the interior of the cavity, with the amount of walking they must do to travel around the interior being linked to their perception of the space’s volume. An ideal nesting cavity is located several meters above ground level, has an interior volume greater than 10 L, and has a southward-facing entrance that is smaller than 30cm² (Seeley, 1977; Franks and Dornhaus, 2003).

Although individual honey bees can distinguish between good and poor quality nesting sites, it is important that all options are considered by a swarm’s scouting bees to ensure that none are overlooked. This balance is achieved by the first scout to visit a potential site and return to the colony having a higher probability of communicating that location to the swarm, regardless of its quality, than subsequent visitors recruited to the site. Scouts also encode the relative quality of nest-site locations via the number of dance circuits they perform. High quality nest sites are reported with more dance circuits per scout per visit compared to mediocre quality site (Seeley and Visscher, 2008). Selection of the best quality nest site is also achieved by more vigorous campaigning for a higher quality nest site as compared to ones of lower quality.
A study by Seeley (2003) demonstrated that scouts who campaigned for the site that was ultimately chosen made more survey trips to that location and danced more upon their return to the swarm cluster than scouts that campaigned for an inferior site that was eventually discarded as an option. On the whole, this indicates that scouts follow a behavioral guideline that stipulates that, for a higher quality nest site, they should make more dance runs after a trip to the potential nest site, they should make more trips, and they should take longer to abandon dancing altogether. Together, these behaviors yield a net effect of exposing more bees to that site as a possibility and obtaining higher rates of recruitment to it (Seeley, 2003).

**Coordination of swarm departure through vibrational cues, piping, and buzz-runs**

The initial departure from the colony or the dissolution of the bivouac and subsequent movement to a new nesting site is a tightly coordinated process (fig. 2). Almost as if a switch was flipped, a quiescent colony is rapidly roused to flight, departing from their original nest or tree branch in one large cloud as the honey bees take to the air in less than a minute. Much effort has focused on understanding the mechanisms that coordinate a tight departure time amongst such a large number of bees. Studies have found that prior to these two fervent departures, from the colony and later on from the bivouac (fig. 2), a cascade of signals is passed between bees to increase their state of excitation and to warn one another of an impending exodus.

In an early study, Martin (1963) noted that the swarm departure from the hive was preceded approximately fifteen minutes by the appearance of buzz runners near the entrance of the nest. During this time, bees run across the comb, fluttering their wings as they zigzag
around their nestmates and break up clusters of workers. Seeley and Rangel (2008) observed similar behavior inside the hive. In the hour preceding departure, the number of buzz-runners was slightly elevated and the frequency of piping signals (a vibrational contact signal passed between workers) increased as liftoff approached. In contrast, no increase in waggle dancing runs or shaking signals were observed over the same periods. Consequently, it is likely that piping signals and buzz-runs are the primary signals for organizing a colony for an impending departure.

In the same vein, preparation for the next large-scale movement of the swarm from the bivouacking site to new nesting site (fig. 2) involves a similar series of signals. One of the most important signals observed on a swarm’s surface prior to bivouac dissolution is the dorso-ventral abdominal vibration, more commonly known as the shaking signal. During this signal, a worker uses her legs to clutch another bee, vibrating the second bee for a period of approximately one second (Visscher et al., 1999). Rather than peaking right in the moments before liftoff, these signals are at their highest frequency between 30 and 60 minutes before departure. Because this signal is observed prior to agreement on a single site, the authors hypothesize that it likely plays a role not only in preparing the swarm for liftoff, but also in motivating scouts to search for and to visit potential nest sites (Visscher et al., 1999). In another study (Gilbert et al., 2011) vibratory workers were removed from the swarm’s surface, but this did not result in a change in the number of sites that were investigated by scouts and recruits or the time it took to select a site for colonization. However, there was a positive correlation between overall vibration signal activity and recruitment dance activity, suggesting that the shaking signal could activate scouts. The time taken for liftoff preparation tripled with the
removal of workers who were engaged in producing shaking signals, suggesting that the primary role of this signal within the bivouac phase of swarming is in informing others to prepare for liftoff (Gilbert et al., 2011).

Another signal employed by honey bees within hives, the piping signal, has also been observed in clustered bivouacs approximately one hour before swarm liftoff to a new nest site. Piping workers travel through the bivouac, intermittently pausing to produce a piping noise by vibrating their wings and pressing their thorax against other workers. These signals are expressed as a pulse of sound with a duration of slightly less than one second, rising in frequency over time. The piping signal stimulates quiet workers to warm up their bodies for flight. This finding was demonstrated by isolating members of the cluster from the piping signals by a screen cage. At the moment of swarm liftoff, these bees remained stationary (Seeley and Tautz, 2001). When plucked from the bivouac and released, they fell to the ground instead of flying, suggesting that their flight muscles were not warm enough for use. These piping signals differ from those expressed in-colony in both their acoustic properties and the body position of workers during piping. Pipers within a bivouachold their wings flat across their back as opposed to the positioning of their wings apart, as is observed within a hive (Seeley and Tautz, 2001). Piping signals are produced exclusively by workers who have engaged in scouting, suggesting that these individuals not only select the new nest site, but are also in charge of the overall preparation of the swarm for liftoff (Visscher and Seeley, 2007).

In addition to piping, the buzz-run is another signal that operates as part of the cascade of signals that induces swarm liftoff. In an early study, Mautz et al. (1972) observed that this
signal assisted in stimulating workers in a queenless cluster to dissolve their bivouac and join a nearby queenright one. Further research has suggested that scouting workers use this signal to disrupt quiescent clusters of bees, having the immediate effect of loosening tight-knit groups (Rittshof and Seeley, 2007). Buzz runners often terminate their signal with a short flight around the cluster before returning to the bivouac and repeating the signal. While piping and shaking signals encourage the swarm to prepare for departure, they also trigger workers to lift off from the cluster as the swarm travels toward its new nest site (Rittshof and Seeley, 2007).

**Airborne guidance of swarms by streaker scouts**

Once a swarm has prepared for liftoff, one of the most striking characteristics of the swarm’s journey is that thousands of the swarm’s individuals are able to successfully move together to the nest site. Two hypotheses have been proposed to explain this phenomenon. In one, Lindauer (1955) observed bees streaking through the airborne swarm at elevated speeds in the direction pointing to the nest site. He proposed that scouts operated as these “streaker” bees, visually guiding the swarm’s travel through their flight paths. The second explanation, proposed by Avitable et al. (1975) suggests that scouts use a pheromone gradient to guide the swarm. In this proposed scenario, referred to as the olfaction hypothesis, scouts emit assembly pheromone from their Nasanov glands on the side of the swarm cloud closest to the nest site, thereby luring the swarm in the correct direction.

A study by Beekman et al. (2006) sought to test these two hypotheses. Analysis of video taken of airborne swarms demonstrated the presence of streaker bees, whose rapid linear paths varied from the slower, more gradual looping flights of ordinary workers within the cloud.
In an experiment where the Nasanov glands of workers within the swarm were sealed, there was no observable affect in the swarm’s ability to navigate to their new nest site, again supporting the vision hypothesis. In a subsequent study by Latty et al. (2009), airborne swarms were subjected to foraging workers flying across their path. The confusion caused by these foraging workers prevented the swarm from successfully travelling to their new nest site en masse. These foragers interfered with the directional information that was provided by streaker scouts, further demonstrating that swarms are visually guided to their final destination by this small number of individuals.

**The potential relationship between worker input and queen’s pheromone production during swarming**

Pheromones produced by the queen play a critical role in maintaining swarm cohesion during swarming. In an early study, Morse (1963) observed that airborne workers within a swarm could be successfully directed by chemicals obtained from crushing a queen’s head, where the pheromone-emitting mandibular glands are located. In the absence of their queen, a swarm of honey bees will lose its cohesive nature. Workers disperse through the area searching for her and will return to the last location where they detect her odor (Butler and Simpson, 1964). For example, in many swarming experiments, the queen is separated from the bivouac within a wire cage (Seeley and Buhrman, 2001; Seeley, 1977; Seeley, 2003). This permits workers to sense her presence through her pheromone production but prevents her from leaving with the swarm during liftoff. Once scouts have chosen a new nest site and the swarm departs without their queen, the airborne workers detect the absence of the queen in flight (presumably by the absence of her pheromones) and will quickly return to the bivouac site.
to recluster around her. In a similar fashion, a swarm that initially departs from the hive without the queen quickly responds to her absence by returning to the colony (Winston, 1987). As the queen represents the only reproductive means for a colony’s success, to depart without her is fruitless and the effort to leave must be abandoned.

In a previous study (Mattila et al.; unpublished data) the queen’s pheromone profile was evaluated using solid phase micro-extraction (SPME) fibers in three distinct colony states: in the colony prior to swarm preparation, at the beginning of the bivouac phase in an artificially induced swarm, and at the moment of swarm liftoff. Preliminary results suggest that queens emit primarily the same compounds in all three settings, but at the moment of swarm liftoff, several new compounds are produced and total pheromone output by the queens is significantly elevated (fig. 3). This increase in pheromone production and the number of components in the blend could potentially serve to advertise her presence to an airborne swarm cloud. It was anecdotally observed during this study that the queen’s activity level was greatly enhanced immediately prior to and during swarm liftoff, which suggests that higher metabolic activity, which may be accompanied by an increase in body temperature, could be responsible for her increase in pheromone output.
Previous studies have also indicated that the queen is subjected to worker signals during the swarming process. A study by Pierce et al. (2006) investigated these signals. Within the colony, in the days preceding swarm issue from the nest, workers directed increasing amounts of vibratory and piping signals toward queens, with signals peaking in the moments before swarm issue. In contrast to this, no vibratory signaling of the queen was observed within the swarm cluster. Piping of the queen was observed within the bivouac in the 2-4 hours preceding...
swarm liftoff, which peaked immediately before departure. It was also noted that, of the workers piping the queen before liftoff, 30-50% were workers who had danced for nest sites. Signaling of the queen by nest-site scouts as they prepare the swarm for liftoff may play a role in providing information to her about when to increase her pheromone output so that she can advertise her presence in the airborne swarm.

**Solid Phase Micro-Extraction as a method for studying queen pheromone production**

Upon discovery of honey bee pheromones, significant effort was directed not only toward understanding their function and role within these insect societies, but also toward analyzing the nature of these chemicals and the quantities in which they were produced. Early methods for determining the chemical components of pheromones in insect systems involved sacrificing subjects and subsequently excising their glands, which were then washed with an organic solvent (Augusto and Valente, 2002). However, sacrificing individuals made it impossible to take repeated samples from single live specimens to analyze pheromone production over time or under different conditions. To address this issue, researchers began experimenting with the use of solid phase micro-extraction (SPME) fibers, a technology developed by Arthur and Pawliszyn (1990). SPME fibers sample the chemicals that are present in the ambient environment and do not require the killing of subjects to accomplish this task. Through the use of gas chromatography coupled with mass spectroscopy (GC/MS), samples can then be analyzed to determine the identity of the compounds and their relative abundance.

In insect systems, SPME technology has been used to sample pheromones through direct contact, where pheromones are collected through the absorbent coating on the fiber
(Monnin et al., 1998) and by exposing the fiber in an organism’s headspace, the air surrounding its body (Zhang and Pawlizyn, 1993). Using these methods, SPME has been used to study a variety of insect systems, including social wasps (Moneti et al., 1998), ants (Monin et al., 1998), and termites (Bordereau et al., 2002). It has also been employed to identify the pheromones that are produced by foragers during waggle dancing (Thom et al., 2007).

**Infrared thermography as a non-invasive method for evaluating temperatures in an insect system**

One way to examine metabolic changes in exothermic insects is to use infrared (IR) technology to monitor body temperatures. Recent advances in IR technology include reduced costs, the development of non-contact sensors, and the availability of more portable imaging units, all of which have allowed the proliferation of IR applications in a variety of fields. Most recently, IR thermography has been applied to the field of animal behavior. An important advantage of IR thermography is its ability to deliver accurate readings in a matter of milliseconds, which allows for temperature readings to be taken from moving targets. Another notable benefit is its ability to measure temperatures without contacting subjects, which reduces the potential of subject distraction or disturbance in an experimental setting. IR thermography is also advantageous because it allows simultaneous readings to be taken on a fine scale, such as differentiating between the surface temperature of an organism’s peripheral limbs and its thoracic region (Kastberger and Reinhold, 2003).

IR thermography has been used in recent years in the area of honey bee biology. Historical methods for determining temperatures in honey bee systems were much more invasive. One method of measuring thoracic temperatures involved anesthetizing the
individual, physically inserting a thermocouple into its thorax via the use of a hypodermic needle, and affixing it in place with resin (Heinrich, 1980). IR thermography has eliminated such disruptive means of data collection. It has also been used to evaluate thermoregulation in wintering honey bee clusters (Stabentheiner et al., 2002), for comparing the thoracic temperature of active and resting bees (Stabentheiner et al., 2003), for evaluating thermal behavior of guard bees when they examine arriving bees (Stabentheiner et al., 2002), for evaluating thermal behavior of honey bees when intruding wasps are balled (Stabentheiner et al., 2007), and for examining how workers within a bivouac warm up prior to swarm liftoff (Seeley et al., 2003).

**Experimental Question**

The aim of the present study was to investigate the relationship between the queen’s pheromone output and her activity level, temperature, and exposure to vibrational signals during the bivouac and liftoff phases of swarming. To accomplish this, swarming behavior was induced in naturally mated honey bee colonies and observational data were collected from queen and workers during the bivouac phase leading up to liftoff. External temperatures of the bivouac’s surface and the queen were measured via infrared thermography. Queen pheromones were measured using a 15-minute exposure to a solid-phase micro extraction fiber, which was later analyzed using gas chromatography/mass spectroscopy to determine component number and quantity. Other parameters of analysis included capturing video data to determine activity level, recording internal bivouac temperatures, and making audio recordings to monitor vibrational piping signals produced in the swarm by workers and received
by the queen. In total, this dataset allowed us to generate a picture of the changes that occur in workers and queens as swarm liftoff approaches, which provides insight into the mechanisms behind the increased pheromone production by queens as they advertise their presence in airborne swarms.

**Materials and Methods**

**Swarm Setup**

In order to determine the relationship between metrics of the swarm and how they may influence the queen’s preparation for the departure toward a new nesting site, a series of swarming trials were conducted on the Wellesley College Campus (Wellesley, MA) between June 1 and September 3, 2011. A total of 16 swarms were prepared, of which 13 yielded usable data. For some data categories, fewer than 13 replicates were produced (see table 1). Of the three swarm replicates from which data were not usable, two replicates failed to cluster around their queen and never formed a cohesive bivouac, and one swarm failed to select a new nest site and never departed from its bivouac site. These swarms were dismantled and returned to their source colonies.
Swarms were prepared from colonies that had been established at Wellesley College’s research apiary in April 2011. All colonies had naturally mated queens that were raised in the previous year. To induce part of each colony to enter a swarming state, approximately 1 kg of workers (~7,500 bees) was shaken from frames and into a large funnel that led to a screened cage. Prior to shaking, the queen was removed from the source colony and placed in a small cage, which was then hung within the bee cluster that formed in the screened cage after shaking. At this point the screened cage and the “swarming” workers it held were transported from the field and into a laboratory setting. For the next 2–3 days (until wax scales formed on the workers’ abdomens—a sign that workers were ready to establish a new home as part of the swarming process), the caged swarm was fed a 50% (v/v) sucrose/water solution that mimicked the high volume of honey that workers consume prior to departing as a swarm from their parental colony. The close quarters of the clustering bivouac in the screened cage, together with the rich food that was provided to the bees during this 3-day confinement period, simulated the swarming experience (Seeley, 1977).

Table 1. Number of swarm replicates from which data in different categories were collected.
Thirteen swarms yielded collectable data, but not all data types were collected from each swarm.

<table>
<thead>
<tr>
<th>Data category</th>
<th>Number of swarm replicates yielding data</th>
</tr>
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<tbody>
<tr>
<td>Surface temperatures of queen and workers</td>
<td>13</td>
</tr>
<tr>
<td>Internal bivouac temperatures</td>
<td>7</td>
</tr>
<tr>
<td>Pheromone profiling of queen</td>
<td>13</td>
</tr>
<tr>
<td>Activity level of queen</td>
<td>13</td>
</tr>
<tr>
<td>Piping signals produced by scouts</td>
<td>9</td>
</tr>
</tbody>
</table>
Following this initial confinement period, the swarm was transferred to a swarm stand where they could cluster around their queen to form an unconfined bivouac from which scouts could issue to search for a new nest site (fig. 4). The queen was first transferred to a small queen cage (7.9 x 3 x 2cm) that was embedded on the board. The cage had a screen on the side that faced the bivouac so that the workers could orient to her for swarm cohesion (via exchange of the pheromones that she produced) and access her for feeding. However, the screen prevented the queen from co-mingling with the workers within the cluster so that observers could gain easy access to her throughout each trial (for data collection) by uncovering the backside of her cage (which was on the bee-free backside of the swarm stand and was covered with cardboard to darken her cage when data were not being collected). A net with a drawstring was attached to the back of the swarm stand so that if the queen left her chamber while the back of her cage was open for data collection, the drawstring could be quickly tightened to prevent her escape. After transferring the queen to the stand, the workers were shaken from the screened cage onto the base of the swarm stand and, eventually (within 30 min.), they migrated to the front of the swarm stand to cluster over their caged queen.
The swarm was allowed to settle into a cohesive bivouac (when the majority of the workers on the bivouac surface were quiescent) before data collection began. The swarm stand was positioned beneath a shade canopy to provide protection from overheating in the sun and to minimize fluctuations in readings of ambient and swarm temperatures with variable exposure to sunlight over the course of each trial. The stand was outfitted with sucrose feeders to provide the bivouac with adequate food supplies so that scouting workers could focus on searching for a new nest site location instead of food sources. Data were collected

Figure 4. A representative image of a swarm stand illustrating placement of queen cage, temperature probes, and microphones for data collection. Two microphones and 8 temperature probes (7 pictured, 1 inserted into the queen cage from the back) were embedded on the swarm stand for data collection.
throughout the nest-site selection process until the bivouac lifted off to depart toward their new home. The embedded queen cage on the stand prevented the queen from leaving with the swarm so that she could be examined at this point in the process. Without the presence of the queen in the airborne swarm, the bees returned to the swarm stand ~5 minutes after lifting off. When all data had been taken from the queen after swarm lift-off and when the workers had returned to the swarm stand (the conclusion of the trial), the entire swarm was returned to its parental colony and a new swarm from a different colony was placed on the swarm stand.

**Internal Bivouac Temperatures**

Internal bivouac temperatures were measured using type K thermocouples (Omega Engineering, Stamford, CT) that were attached to various points on the face of the swarm stand. The tip of each probe was positioned to protrude approximately 1.5cm perpendicular to the surface of the swarm stand so that they could measure temperatures several bee layers into the bivouac. Three probes were affixed over the queen’s cage (2 in front, 1 in back); four other probes were positioned within the bivouac (see fig. 4 for probe placement), and one more probe was placed on the edge of the swarm stand, approximately 20cm away from the bivouac, to measure ambient temperature. Temperatures were recorded from each probe every minute by thermometer data loggers (Omega, model HH309A, Stamford, CT; accuracy: ±0.2% of reading).
Surface Temperatures of Workers in Bivouac and of Queens

Surface temperatures of each bivouac and its queen were measured hourly via infrared (IR) thermography with a thermographic camera (FLIR Systems, model T300, Wilsonville, OR; thermal sensitivity: <0.05°C; accuracy: ±2°C or ±2% of reading). The emissivity was set to 0.97, which is the emissivity of an insect’s cuticle (Stabentheiner and Schmaranzer 1987, Kovac and Stabentheiner, 1999) and measures of the ability of a surface to emit radiation energy, with values ranging between 0 and 1 (dimensionless quantity). Emissivity is inversely proportional to reflectivity, so the duller a material is, the higher is its emissivity. A bee cuticle falls on the dull end of the spectrum, with an emissivity value of 0.97 (Stabentheiner and Schmaranzer 1987, Kovac and Stabentheiner, 1999). Setting the emissivity informs the camera how much of the energy is being radiated by the object of interest compared to that emitted by objects in the surrounding environment and reflected off the object of interest (Stabentheiner and Schmaranzer 1987, Kovac and Stabentheiner, 1999). Prior to the collection of each hourly dataset, the camera settings were adjusted for ambient temperature, relative humidity, and reflective apparent temperature. Ambient temperature and relative humidity were measured with an Ambient Weather Station (Model WS-0101, Ambient Weather, Chandler, AZ; humidity accuracy: ±5%; temperature accuracy: ±2°F). Reflected apparent temperature is a measure of how much energy is being reflected off of the object of interest and into the camera as opposed to radiated from it, which is the measure we were interested in obtaining. Setting an accurate reflected apparent temperature is necessary for obtaining accurate temperature readings (FLIR Systems, 2010). Reflected apparent temperature was determined by setting the emissivity of the camera to 1.00, then placing an aluminum foil diffuse infrared reflector below the bivouac
on the swarm stand and taking a spot measurement of the temperature on the reflector.

Temperature readings of focal subjects (bivouacs or queens) fluctuated slightly from image to image, so three images were taken sequentially at each time point and temperature readings were averaged across the three images to produce a single temperature value for that time point.

One challenge that was posed when taking thermographic images of a queen on a stand was the potential for her to escape from her cage when the back of it was opened for unobstructed infrared imaging. This risk was minimal when the queen was not that active, but at times her activity level was higher and there was a good possibility of her escape. To prevent her from escaping when thermographic images of her were made, FDA-grade, 75-gauge, clear polyolefin film was used to cover the backside of the queen’s cage (Polyolefin shrink wrap film, Uline, Waukegan, IL). Sadler and Nieh (2011) utilized a similar film to cover two sides of an observation hive while collecting thermographic measurements of waggle-dancing honey bees. This material allows infrared waves to travel through the film without obstructing the subjects behind it. As shooting thermographic images through the film does significantly decrease the temperatures of the subjects it covers, Sadler and Nieh (2011) found it necessary to calculate a correction factor to adjust subject temperatures to their actual values. This method was adapted for the purpose of this study. A special backing for the queen cage was prepared with a solid outer frame and an infrared transmissive film suspended across it that provided a barrier that served as the back of the queen’s cage. During periods of low activity, images were taken under two conditions: with an unobstructed back and through the infrared transmissive film. The infrared transmissive film significantly depressed temperature readings of the
queens’ thoraxes (paired t-test; t=0.92; df=14; p=0.023). To correct for this reduction, a linear regression was performed between the temperature readings that were taken with an unobstructed view of the queen and those taken through the film to yield a correction-factor equation: \( \text{Temp}_{\text{corrected}} = 0.871(\text{Temp}_{\text{uncorrected}}) + 5.143 \) \((n=155 \text{ data points}; r^2 = 0.89)\). For the majority of temperature readings, images that were taken with an open back were used. For data points where images could only be taken through film due to increased activity of the queen, temperatures were corrected using this equation.

Temperature data were extracted from the thermographic images (FLIR QuickReport software, FLIR Systems, Wilsonville, OR) by drawing a box over the area of interest on the image and averaging the temperature within this area. For the queen, the box was drawn over her thorax. Average temperatures were measured over the surface of the swarm for: 1) the full bivouac surface, 2) the surface area over the queen’s cage (which was marked by screws that were attached to swarm stand above and below her cage that were visible in the thermographic images), and 3) an area immediately adjacent to the queen cage where workers were present in similar quantities (i.e., two-dimensional density and number of bee layers).

Two methods have been widely used when thermographic IR measurements are taken of honey bees: either taking the absolute temperature of the honey bees (Seeley, et al., 2003; Stabentheiner et al., 2002; Stabentheiner et al., 2007) or calculating the temperature of the honey bees relative to ambient temperature (Mapalad, et al., 2008; Sadler and Nieh, 2011). Insects are generally regarded as exothermic (i.e., cold-blooded) organisms. However, individual bees show a remarkable ability to control their body temperature through repeated
contraction of their thoracic muscles (Winston, 1987), to the extent that the colony as a collective can be considered endothermic. Honey bees can also lower the temperature of the collective colony relative to ambient through such behaviors as fanning and spreading water to dissipate heat through evaporative water loss (the same way that humans sweat). In this study, we chose to use absolute temperature because a critical element in preparing a swarm for departure is that all workers and the queen have a flight-ready thoracic temperature that is greater than 35.0°C. In general, surface temperatures of bivouacking workers and queens rose from morning to afternoon as ambient temperature climbed. However, swarms departed anywhere from 8:00 AM to 4:30 PM, so estimates of changes in the temperatures of workers and queens relative to ambient would have been confounded by differences in time of day, whereas absolute temperature provided a better picture of how possible flight was for swarming workers and queens as liftoff approached.

Activity Level

Activity level of each swarm’s queen was estimated based on a video clip that was taken of her on an hourly basis through the transparent film on the backside of her queen cage (Sony HandyCam, model DCR-HC62 digital video camera, Tokyo, Japan). The activity level of the queen was measured over one minute by laying a transparency over the image of the queen’s cage on the computer monitor and tracing her path as the video played and she moved within her cage (videos were replayed frame by frame in Final Cut Express 4.0.1, Apple Inc., Cupertino, CA). The transparency was scanned and imported into Adobe Illustrator CS5 (Adobe Systems, San Jose, CA), where the queen’s path was traced with the pen tool to determine path length (cm).
each video recording, pathdistance was scaled relative to the actual size of the queen cage in
the image to account for differences in image magnification based on the position of the
camera across time points.

Piping Signals

Piping signals, which are produced by nest-site scouts to warn workers to warm up their
flight muscles for impending lift-off, were measured hourly via audio recordings that were made
within the bivouac with a digital voice recorder (Olympus America Inc., model WS-600S, Center
Valley, PA). Two omni-directional tie-clip microphones (frequency response 50-16,000Hz,
sensitivity: -65dB ± 3dB; RadioShack Corporation, Fortworth, TX) were embedded in the bivouac
for this purpose by affixing them to the swarm stand (see fig. 4 for microphone placement). For
each audio recording, the number of piping signals that was heard during a one-minute interval
was determined. For time points that were close to liftoff, piping signals were almost continual,
with several scouts producing signals concurrently. At these time points, it became impossible
to count individual piping signals, so a value of 60 piping signals per minute was assigned for
the purpose of statistical analyses, although this is likely a gross underestimation of the actual
number of piping signals that were produced.

Pheromone Sampling

The pheromone profile of each queen was measured by exposing her in a closed
container to 65µm polydimethylsiloxane-divinyl benzene (PDMS-DVB) solid-phase micro
extraction (SPME) fibers (Sigma Aldrich, St. Louis, MO). The PDMS-DVB coating was identified
by Thom et al. (2007) as having a heightened ability to absorb non-polar molecules, the type of compound that is present in queen pheromone emissions. Each sampling was carried out by removing the queen from her cage on the swarm stand and placing her in a 100mL glass jar “chamber” to sample the pheromones in her headspace. Prior to sampling, each chamber was cleaned in an attempt to rid it of contaminants. Chambers were washed with an Alconox cleaning agent (VWR International, West Chester, PA), acetone, and hexane, with a drying period between each wash. After the hexane rinse, chambers were inverted and allowed to air out over night prior to use and then their openings were sealed with foil caps to prevent environmental contamination. This cap had an embedded cylindrical cage protruding down into the chamber that prevented the queen from touching the exposed fiber during sampling.

Before a queen was sampled in the chamber, a “blank” was taken by puncturing the foil seal and injecting a SPME fiber into the chamber for 15 minutes to sample any compounds that were in the empty chamber; these compounds were later subtracted from the range of compounds that were sampled from the chamber when it held a queen. After the collection of a blank, the queen was transferred to the testing chamber. The chamber was wrapped in foil to mimic the darkened conditions that the queen would experience within the swarm cluster, unless the sampling point was at liftoff—then the chamber was left uncovered because the queen would be flying through the air at that point in the natural swarming process. Once the queen was in the chamber, a SPME fiber was injected into it for 15 minutes to collect a pheromone sample of her headspace.

On days when a swarm did not liftoff, pheromone sampling was conducted once in the morning and once in the late afternoon. On the day of swarm liftoff, sampling was carried out
once in the morning and at the moment of swarm lift, barring circumstances where signs of that the swarm would liftoff early in the morning were observed. In this latter instance, to remove the queen from the bivouac risked the possibility of the swarm departing while we were sampling, so queens were only sampled when the bivouac dissolved to travel toward its new nest site.

Gas chromatography/mass spectroscopy (GC/MS) was employed to separate and quantify the compounds that were present in each headspace sample from a queen. Pheromone samples captured with SPME fibers were injected into a Hewlett Packard 5890 Series II Gas Chromatograph with a HP-5ms, 30m, 0.025m, interior diameter column. Each SPME fiber sample was injected into the column at an injection temperature of 240°C over the course of 3 minutes. The column was maintained at an initial temperature of 40°C for 3 minutes and then cycled upwards at a rate of 15°C per minute until it reached a maximum of 300°C. The temperature was maintained at 300°C for the final 5 minutes of the run. The flow rate of the helium carrier gas was 1 ml/minute.

Samples were subsequently analyzed using a Hewlett Packard 5972 Mass Selective Detector (GMI Inc., Ramsey, MN). Gas chromatography allows for the separation of compounds via their physical properties (Mohrig et al, 2006). A headspace sample on a fiber was manually injected into the unit, where its components moved through the column by an inert gas (helium in this instance). Compounds that interact with the material that coats the column and compounds with lower boiling points move through the column faster, yielding lower retention times. On the opposite end of the spectrum, compounds that fail to interact with the column
coating or have higher boiling points move through the column more slowly and have higher retention times. The resulting data are expressed in a gas chromatograph, a graph with retention time on the x-axis and relative compound abundance (abundance of molecules in the sample corresponding to that retention time) on the y-axis. Discrete peaks on the x-axis represent the different compounds, with the height of that peak indicating how much of that compound was present in the sample (Mohrig et al, 2006).

To differentiate peaks that belonged to the queen’s pheromone profile from peaks that belonged to compounds that were present in the sampling chamber or the environment, samples were first examined on a per-swarm basis. The gas chromatography data for all blanks within a given swarm were overlaid in ChemStation (Build D.00.00.38, Agilent Technologies Inc., Santa Clara, CA) and a list was generated of retention times for which peaks were observed in at least 75% of the blank samples for that swarm at an abundance greater than 1,000 counts. These were designated as blank peaks and were disregarded in the chromatographs that were generated from the samples of compounds that were emitted by the queen. All queen samples for the same swarm were overlaid and the aforementioned procedure was repeated, which generated a master list of retention times for peaks that were deemed to belong to the queen’s pheromone profile (fig. 5).

With a master list of retention times for compounds that were either commonly observed across blanks (to be disregarded) or in the queen’s pheromone profile, chromatographs for each time point in a swarm replicate were then analyzed individually. For each time point, the queen and blank gas chromatographs were overlaid. A list of peaks that
were designated as corresponding to queen compounds was generated for that time point according to the following criteria: peaks in the queen chromatograph had an abundance greater than 1,000 counts, the peaks were on the “master list” of queen peaks for that swarm replicate (as described above), and the peak was observed in the queen sample but not the blank (fig. 5). This method excluded from consideration peaks that were produced at only certain time points in the swarming process (i.e., they were not observed commonly across chromatographs). However, previous work has suggested that honey bee queens produce novel compounds as swarms lift off (Mattila et al.; unpublished data). To account for novel peaks that a queen might emit only as liftoff approached, peaks were also counted as part of a queen’s pheromone profile in samples approaching liftoff if the peak was observed in the queen sample but not the blank and it had an abundance greater than 2,500 counts. A more conservative abundance criterion (minimum 2,500 counts for novel peaks rather than 1,000 counts for common peaks) was used to avoid counting small peaks that were part of a noisy baseline as actual queen compounds.
Figure 5. Sample procedure for identification of peaks representing compounds emitted by the queen. **A)** All blanks for a swarm were overlaid on a colony scale and master list of “blank peaks” created that would be excluded from consideration. **B)** All queen samples for a swarm were overlaid and a master list made of “queen peaks” generated for novel peaks that appeared in the queen samples but not the blanks. **C)** Samples were then analyzed on an individual basis. Peaks of interest met the following criteria: appeared on the master list of queen peaks for that swarm, had an abundance greater than 1,000 counts, and, for that time point, had a peak for the queen sample not a corresponding blank peak (example, no red blank peak below the blue queen peak, suggesting that this compound peak was not an environmental component). These chosen peaks (example: retention time of 8.43 in sample C) were integrated and used for further data analysis of pheromone production by the queen.
While 6 of the 13 swarms yielded a clean baseline in their gas chromatography data, samples from seven swarms suggested the presence of a large hydrocarbon contaminant on the GC/MS coil. For these swarms, the baseline was much noisier, making it difficult to differentiate significant peaks from background noise. For this reason, the lists of queen peaks for the six swarms with clean baselines were combined to create a master list of all retention times of interest (i.e., peaks to look for in the swarms that had noisier baselines). To be counted as a queen peak for the noisier swarm chromatographs (novel peaks or peaks present on the master list of queen peaks for that swarm), the retention time had to be present on the master list of retention times of interest that was generated from the six swarms with clean baselines.

Once peaks of interest had been identified in each sample, the area under each peak was integrated using the RTE algorithm in ChemStation (Version B.01.00, Agilent Technologies Inc., Santa Clara, CA) to determine the relative amount of each compound (i.e., area under a peak) that was emitted by the queen during a given time point. For each time point in a swarm replicate, the integration values for all queen peaks were combined to provide a single integration value that represented the total amount of pheromones that was emitted by the queen during that sampling time point.

Data Analysis

The experimental unit for this study was a swarm. Therefore, to avoid pseudo replication, data points taken repeatedly from a swarm during different phases of the swarming process were collapsed to produce a “phase” data point. These phases were identified as: phase 0) bivouac setup on the swarm stand; phase 1) non-liftoff day or up to 1 hour before
lift off, phase 2) within 5-60 minutes of liftoff, and phase 3) within 5 minutes of liftoff. Data from each phase of all swarm replicates were used in one-way analyses of variance (ANOVA’s) to determine whether swarm and queen metrics changed significantly as liftoff approached. Data types for which this analysis was conducted includes activity level of queens, number of unique compounds present and total pheromone output for their pheromone profiles, frequency of piping signals produced by workers, and surface temperatures of bivouacs and queens. While 13 swarms yielded usable data, not all data types/phases had this many replicates for a variety of reasons (see table 2 for an explanation of how many replicates were obtained for phase averages for each data type). An average temperature on the bivouac surface in two locations: directly over the queen and in an area immediate adjacent to her location in the bivouac were calculated for each swarm. These values were used in a paired t-test to determine whether workers selectively heated the area of the bivouac immediately over the queen as opposed to the area adjacent to the queen's location. Spearman correlations explored relationships between metrics of queens and bivouacs approaching swarm liftoff and used raw data values as opposed to phase averages. Data analyses were conducted in SAS (Version 9.1, SAS Institute Inc., Cary, NC).
Table 2. The number of swarm replicates yielding data for ANOVA calculations by data type and phase of the swarming process. Although thirteen swarms yielded collectable data, there was variation in what types of data were collected during each phase of the swarming process due to the natural unpredictability of swarm liftoff and other reasons. Within each swarm, individual time points for each data type were collapsed into a phase average to avoid pseudo-replication. The below table summarizes how many swarm replicates went into the ANOVA tests, means, and standard error calculations for each data type at all phases of the swarming process in the format: n=# replicates obtained/number of swarms for which that data type exists. Unless otherwise indicated, replicates are absent either due to the natural unpredictability of the swarm liftoff (example – swarm liftoff is a sudden process, often taking less than a minute and sometimes enough warning was not given to have equipment prepared for data collection at lift) or a phase is absent for one or more swarms due to the nature of the timeline followed by the swarm approaching liftoff (example – some swarms left early in the morning right as data collection was beginning for the day, leading to an absence for data points in the phase within 5-60 minutes of liftoff). For data types/phases where there are other reasons for a lessened or absent number of data points, the reason is indicated.

**Explanation A)** When the swarm appeared to be preparing for an impending departure, the queen was not removed from the bivouac for pheromone sampling lest the swarm depart during the sampling, thus losing the opportunity for data collection from the queen at liftoff.

**Explanation B)** Activity level data collection began once the swarm had settled and was not taken at bivouac set-up. **Explanation C)** The window of time for liftoff was short, priority was placed on obtaining infrared images of the queen and commencing pheromone sampling over delaying these types of data collection by several minutes to obtain a video for activity level calculations. **Explanation D)** A settling period was needed for the swarm to form a cohesive bivouac before surface temperature data collection could begin. **Explanation E)** Piping signals were not produced until after the swarm had settled and the nest selection process was well underway.

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<th>Within 5-60 minutes of liftoff</th>
<th>Within 5 minutes of liftoff</th>
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<td>n=6/13</td>
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<td>n=10/13</td>
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<td>n=5/9</td>
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</table>
Results

Queen and worker temperatures rose as liftoff approached

Changes in the temperatures of both bivouacking workers and queens suggest that they both significantly increase their thoracic temperatures in the minutes leading up to liftoff (figs. 6, 7, 8). The temperature of the complete bivouac surface (one-way ANOVA, $F=4.8$; df=2,34; $p=0.01$) the bivouac in the area directly over the queen (one-way ANOVA, $F=3.5$; df=2,34; $p=0.04$), and the bivouac in an area adjacent to the queen chamber (one-way ANOVA, $F=4.0$; df=2,34; $p=0.03$) remained relatively consistent during the nest-selection process, did not begin to rise until the hour before liftoff, and peaked within 5 minutes of swarm liftoff (figs. 6, 7, 8).

Similarly, the queen’s thoracic temperature did not significantly vary between bivouac set-up and 5 minutes pre-liftoff, significantly spiking in those 5 minutes before swarm departure (one-way ANOVA, $F=8.3$; df=3,44; $p=0.002$; figs. 6A, 8). One unexpected trend that was observed was that the queen’s temperature was similarly high at bivouac set-up as it was during swarm liftoff (fig. 6A), which may be an artifact of handling the queen as she was transferred to the swarm stand. This disturbance may have antagonized her, leading to an increase in mean thoracic temperature at this point in the process.

Bivouacking workers selectively heated queens

To evaluate whether the workers within the bivouac were selectively heating the queen, temperatures between two locations on the bivouac surface (directly over the queen and in an area immediately adjacent to the queen’s location) were compared. Workers maintained higher
surface temperatures in the area directly over the queen compared to the neighboring area of the bivouac where workers were present in similar quantities, but were not in direct proximity to the queen (paired t-test, t = 3.0; df = 12; p=0.01; fig. 9).

**Piping signals from scouts increased as liftoff approached**

Piping signals that were produced by workers in the bivouac were evaluated across the different phases of swarming to determine whether they increased as swarm liftoff approached. No significant differences were found between the number of piping signals recorded by the two microphones that were positioned on the swarm stand (one near the queen cage and the other at the top of the swarm stand; paired t-test, t=1.4; df=100; p=0.16), so the two values from each microphone were averaged to yield a single mean value per time point. Piping signals increased significantly in the hour before liftoff compared to prior time points and peaked in the five minutes before swarm departure (one way ANOVA, F=22.8; df=2,19; p<0.0001; fig. 10).

**Queens were not definitively more active as swarm liftoff approached**

A comparison of the activity level of queens across the different phases of the swarming process showed a non-significant trend toward increased queen activity as swarm liftoff approached (one way ANOVA, F=2.5; df=2,28; p=0.10; fig. 11). However, great variability in the activity level of queens at the moment of liftoff potentially masked real differences among swarm phases. Activity levels of queens ranged widely, with values observed as low as 0.5cm/min and as high as 82.5 cm/min. Of the 6 queens whose activity levels were evaluated at
liftoff, results were polarized: 2 queens moved at speeds lower than 1 cm/min, 2 queens moved at speeds greater than 60 cm/min, and 2 queens had intermediate activity levels. Further swarm replicates may solidify conclusions regarding the role that activity level plays in modulating queen physiology and behavior as swarms liftoff.

**Pheromone production did not significantly increase as swarm liftoff approached**

No significant differences were observed in total pheromone emission by queens across different phases of the swarming process (one-way ANOVA, F=0.12; df=2,3; p=0.89; fig. 12A). Similarly, there was no difference in the discrete number of compounds (i.e., peaks in the gas chromatograms) that were emitted by the queen as the swarm approached liftoff (one-way ANOVA, F=0.44; df=2,35; p =0.65; fig 12B). As with activity level, an increasing trend over time was diluted by a large degree of variability within each time point (fig. 12). The results of this study contrast to the findings of a study conducted the previous year (Mattila et al.; unpublished data) and may be related to differences in methods of queen handling and sampling intervals between the two studies (see Discussion).

**Queens heated up as bivouacs heated up**

The surface temperatures of bivouacs and their queens were compared to evaluate whether these parameters rose together as swarms neared liftoff. A positive correlation was observed between the mean surface temperature of the entire bivouac and the surface temperature of the bivouac directly over the queen (table 3; fig. 13A).The mean temperature of
the entire swarm face and the bivouac over the queen correlated positively with the temperature of the queen (table 3; fig. 13B, 13C), suggesting their temperatures rose together.

**Increases in piping signals are not reliably linked to temperature changes in bivouacking workers and queens**

Piping signals produced within the bivouac were correlated against temperatures for queens and bivouacking workers to determine whether piping was linked to physiological changes in workers and queens as swarming approached. While the relationship between an increase in piping signals and an increase in mean temperature across the entire bivouac surface was established only marginally (i.e., at the level of significance; table 3; fig. 14A), a positive correlation was observed between the production of piping signals and the mean temperature of the bivouac in the region directly over the queen (table 3; fig. 14B). No significant correlation was observed between piping signals produced by scouts and the thoracic temperature of the queen (table 3; fig. 14C), suggesting a less important role for piping in preparing queens for liftoff.

**The activity level of queens increased as bivouacs and queens heated up, but not in response to increased piping signals**

Piping signals produced by workers were compared to the activity level of their queens to determine whether the latter’s activity level increased as the bivouac prepared for an impending swarm departure. No significant correlation was found between piping signals and the activity level of the queen (table 3; fig. 15A). However, it was clear that the queen’s activity level was positively correlated with the temperature of both the entire bivouac (table 3; figure 15B) and in the region of the bivouac directly over the queen (table 3; figure 15C). Similarly,
the thoracic temperature of the queen was also positively correlated with her activity level (table 3; figure 15D).

**Queens emit more pheromones as piping signals increased**

Piping signals produced within bivouacs were compared to the pheromone production by queens to determine whether information from workers about liftoff was linked to pheromone production by queens. A positive correlation was observed between piping signals and total pheromone production by queens (table 3; fig. 16A). In contrast, the number of pheromonal compounds that were emitted by queens did not increase as piping signals became more frequent (table 3; fig. 16B).

**Pheromone emission by queens was tied to swarm temperature but not queen activity**

Activity levels of queens were compared against their pheromone production to determine whether queen speed, presumed to accompany an increase in metabolic activity, played a role in the physiological processes that result in increased pheromone output by queens at the time of liftoff. However, the activity level of queens did not affect her relative pheromone output or the number of pheromonal compounds she emitted (table 3; figs. 17A, 17B), which suggests that a change in activity level is not associated with the mechanisms that lead to an increase in pheromone production by queens.

In general, the greater the total pheromone emitted by a queen, the more unique compounds were present in her pheromone sample (table 3; fig. 18). Interestingly, total pheromone emission by queens was significantly and positively correlated with mean temperature across the surface of bivouacs and the mean temperature across bivouacs in the
region directly over queens (table 3; figs. 19A, 19C). Similarly, the mean number of pheromonal compounds that were emitted by queens increased significantly with increases in the mean temperatures across the complete bivouac surface and in the region directly over the queen (table 3; figs. 19B, 19D). The total pheromone output of the queen was also found to positively correlate with her thoracic temperature (table 3; fig. 19E). In contrast, the thoracic temperature of the queen failed to show a significant relationship to the number of compounds that she emitted (table 3; fig. 19F). Even considering this one contradictory relationship, the results overwhelmingly indicate that the pheromone output of queens, in terms of both amount of pheromone and number of unique chemicals that were emitted, was enhanced by an increase in the temperature of both the bivouac and queen themselves.
Figure 6. The surface temperatures of both queen and swarm increase approaching swarm liftoff toward a new nest site. Temperatures were measured hourly via infrared photography over the course of the swarming process. Utilizing specialized software, an area box was drawn over the area of interest on each image (queen’s thorax, entire bivouac surface, or bivouac surface in region located directly over the queen) and the mean temperature within that area determined. Temperatures were averaged within each swarm and phase to avoid pseudo-replication. One way ANOVA tests were used to compare the temperatures across the different phases. See table 2 for a summary of how many replicates went into each data type/phase average. Tukey letters denote significant difference. Error bars represent the standard error of the mean. A) The mean thoracic temperature of the queen significantly increases approaching swarm liftoff. B) The mean surface temperature of the complete swarm face significantly increases approaching swarm liftoff. C) The mean surface temperature of the bivouac in the area located immediately over the queen significantly increases approaching swarm liftoff. D) The mean surface temperature of the swarm face in the area immediately adjacent to the queen chamber within the bivouac significantly increases approaching swarm liftoff.
Representative photograph of a bivouac on the swarm stand. 

Infrared image - 70 minutes pre-liftoff
Mean temperature across bivouac surface: 31.9°C

Infrared image – 17 minutes pre-liftoff
Mean temperature across bivouac surface: 32.4°C

Infrared image – at moment of liftoff
Mean temperature across bivouac surface: 35.3°C

Figure 7. The surface temperature of the bivouac significantly increases approaching swarm liftoff. Temperatures were measured via infrared photography hourly over the course of the swarming process. Utilizing FLIR QuickReport software, the mean temperature across the swarm’s surface determined. Infrared images shown above were obtained from the same swarm as it approached liftoff.
Figure 8. The thoracic temperature of the queen significantly increases approaching swarm liftoff. Temperatures were measured via infrared photography hourly over the course of the swarming process. Infrared images shown above were obtained from the same queen/swarm as they approached liftoff. White arrows indicate the position of the queen’s thorax in each image. The images taken 70 minutes prior to and at the moment of liftoff were taken through an infrared transmissive film to prevent the active queen from escaping from her chamber. The displayed thoracic temperatures for these two images have been corrected using the equation: Temp_{corrected} = 0.871(Temp_{uncorrected}) + 5.143 (n=155 data points, R^2=0.885).
Figure 9. The workers in the bivouac selectively heat the area immediately over the queen. Temperatures were measured hourly via infrared photography over the course of the swarming process. Utilizing specialized software, an area box was drawn over two areas on the swarm surface to evaluate whether the workers were selectively heating the queen: the area immediately over the queen and the area adjacent to this region where workers were present in similar abundance. Workers selectively heat the queen, with the hottest temperatures observed directly over her location within the bivouac. Error bars represent the standard error of the mean; an asterisk denotes a statistically significant difference.
Figure 10. The piping signals produced by scouts to warn quiescent workers within the cluster of an impending liftoff significantly increase approaching swarm liftoff. Piping signals were recorded via microphones embedded on the swarm stand within the bivouac in two locations: near the queen cage and in another area of the bivouac where the bees were present in highest quantities. No significant difference was observed between piping signals recorded at the two locations within the cluster, leading to the averaging of these two values to yield a single mean value per time point. A one way ANOVA was used to evaluate significant differences between piping signals produced during the different phases. Tukey letters denote significant difference between different phases of the swarming process, error bars represent the standard error of the mean. See table 2 for a summary of how many swarm replicates were obtained for each phase for calculation of means and ANOVA tests.
Figure 11. The queen’s activity level does not significantly increase approaching swarm liftoff. The activity level of the queen was measured hourly throughout the swarming process via video recording of her movements within her chamber on the swarm stand. Videos were imported to FinalCut Express, a transparency overlaid on the screen, and the path each queen took over the course of one minute was traced. Transparencies were scanned into the computer, imported into Adobe Illustrator CS5, the path traced with the pen tool, and the path length determined. Path length was converted into speed. A one way ANOVA was used to evaluate significant differences between activity level of the queen during the different phases of the swarming process. No significant differences were observed in the queen’s activity level between the various phases within the swarming process. See table 2 for a summary of how many swarm replicates were obtained for each phase type for calculation of means and ANOVA tests.
Figure 12. The queen’s pheromone production does not significantly increase approaching swarm liftoff. Pheromone profiling was executed via exposure of a solid phase micro-extraction (SPME) fiber in the queen’s headspace. A blank was first obtained by exposing a fiber in the glass sampling chamber for 15 minutes. The queen was then transferred to the chamber and a new fiber exposed for a 15 minute sampling period to pick up a sample of the pheromones being emitted by the queen. Fibers were then analyzed using gas chromatography/mass spectroscopy (GC/MS) to separate the pheromone sample into its individual components and determine the relative abundance of each compound in that sample. Samples were analyzed on a per swarm basis to distinguish compounds belonging to the queen from ones naturally present in the sampling environment. Peaks belonging to the queen were integrated and a sum calculated for each sample to give a total integration value for each sample representing the relative amount of pheromone being emitted by the queen. One way ANOVA’s were conducted to evaluate differences between phases for each data type. See table 2 for a summary of how many swarm replicates were obtained for each phase for calculation of means and ANOVA tests. A) The relative amount of pheromones (represented by the total integration value) being emitted by the queen does not significantly increase approaching swarm liftoff. B) The mean number of distinct compounds being produced by the queen (represented by number of peaks) does not significantly increase nearing swarm liftoff.
Table 3. Spearman correlations between metrics measured for the bivouacking workers and queens. Significant differences are marked with asterisks: * indicates p<0.05, ** indicates p<0.01, *** indicates p<0.001.

<table>
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<tr>
<th></th>
<th>Piping signals produced by workers (#/min)</th>
<th>Mean temperature across the entire bivouac surface (°C)</th>
<th>Mean temperature across the bivouac surface directly over the queen (°C)</th>
<th>Thoracic temperature of the queen (°C)</th>
<th>Activity level of the queen (cm/min)</th>
<th>Total integration value per pheromone sample (abundance)</th>
<th>Total number of peaks per pheromone sample</th>
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Figure 13. The correlative relationships between the temperature of the entire bivouac surface, the bivouac surface directly over the queen, and the thoracic temperature of the queen. Temperatures were measured hourly via infrared photography over the course of the swarming process. Utilizing specialized software, an area box was drawn over the area of interest on each image (queen’s thorax, entire bivouac surface, or bivouac surface in region located directly over the queen) and the mean temperature within that area determined. Temperatures were correlated against one another using Spearman Correlations. See table 3 for correlation coefficients, p values, and sample sizes. A) The mean temperature across the entire bivouac surface positively correlates with the mean temperature across the bivouac surface directly over the queen. B) The mean temperature across the entire bivouac surface positively correlates with the thoracic temperature of the queen. C) The mean temperature of the bivouac in the region directly over the queen positively correlates with the thoracic temperature of the queen.
Figure 14. The correlative relationship between piping signals produced within the bivouac and the surface temperatures of the bivouac and queen. Piping signals were recorded via microphones embedded on the swarm stand within the bivouac in two locations: near the queen cage and in another area of the bivouac where the bees were present in highest quantities. No significant difference was observed between piping signals recorded at the two locations within the cluster (paired t-test, t = 1.41; df = 100; p=0.163), leading to the averaging of these two values to yield a single mean value per time point. Surface temperatures of queen and bivouac were measured via infrared photography. Utilizing specialized software, an area box was drawn over the area of interest on each image (queen’s thorax, entire bivouac surface, or bivouac surface in region located directly over the queen) and the mean temperature within that area determined. Spearman correlations were used to evaluate relationships between piping signals produced by workers and the surface temperatures of the bivouac and queen. See table 3 for correlation coefficients, p values, and sample sizes. A) The piping signals produced by scouts positively correlate with the mean temperature across the entire bivouac surface at the level of significance. B) The piping signals produced by scouts positively correlate with the mean temperature across the bivouac surface directly over the queen. C) The piping signals produced by scouts fail to correlate with the thoracic temperature of the queen.
Figure 15. The correlative relationships between the queen’s activity level, piping signals produced by scouts, and the surface temperatures of the queen and bivouac. The activity level of the queen was measured hourly throughout the swarming process via video recording of her movements within her chamber on the swarm stand. Videos were imported to the computer, a transparency overlaid over the screen, and the path she took over the course of one minute was traced. Transparencies were scanned into the computer, imported to Adobe Illustrator CS5, the path traced with the pen tool, and the path length determined. This was converted into an activity level, measured as speed. Piping signals were recorded via microphones embedded on the swarm stand within the bivouac in two locations: near the queen cage and in another area of the bivouac where the bees were present in highest quantities. No significant difference was observed between piping signals recorded at the two locations within the cluster (paired t-test, \( t = 1.4; \) df = 100; p=0.163), leading to the averaging of these two values to yield a single mean value per time point. Temperatures were measured hourly via infrared photography over the course of the swarming process. Utilizing specialized software, an area box was drawn over the area of interest on each image (queen’s thorax, entire bivouac surface, or bivouac surface in region located directly over the queen) and the mean temperature within that area determined. Spearman correlations were used to evaluate relationships between activity level and the surface temperatures of the swarm and queen. See table 3 for correlation coefficients, p values, and sample sizes. A) The piping signals produced by scouts fail to correlate with the activity level of the queen. B) The mean temperature across the entire bivouac surface positively correlates with the activity level of the queen. C) The mean temperature across the bivouac surface in the region directly over the queen positively correlates with the activity level of the queen. D) The thoracic temperature of the queen positively correlates with her activity level.
Figure 16. The relationship between the piping signals produced by scouts and the pheromone production of the queen. Pheromone profiling was executed via exposure of a solid phase micro-extraction (SPME) fiber in the queen’s headspace. A blank was first obtained by exposing a fiber in the glass sampling chamber for 15 minutes. The queen was then transferred to the chamber and a new fiber exposed for a 15 minute sampling period to pick up a sample of the pheromones that she was emitting. Fibers were then analyzed using gas chromatography/mass spectroscopy (GC/MS) to separate the pheromone sample into its individual components and determine the relative abundance of each compound in that sample. Samples were analyzed on a per swarm basis to distinguish compounds belonging to the queen from ones naturally present in the sampling environment. Peaks belonging to the queen were integrated and a sum calculated for each sample to give a total integration value for each sample representing the relative amount of pheromone being emitted by the queen. A sum of the peaks belonging to the queen was also determined to represent the total number of discrete pheromonal compounds being released by the queen. Spearman correlations were used to evaluate relationships between piping signals produced by workers and the pheromone production of the queen. See table 3 for correlation coefficients, p values, and sample sizes. A) The piping signals produced by scouts positively correlate with the amount of pheromone being emitted by the queen. B) The piping signals produced by the scouts positively correlate with the number of pheromonal compounds being secreted by the queen.
Figure 17. The activity level and pheromone production of the queen fail to correlate.

Pheromone profiling was executed via exposure of a solid phase micro-extraction (SPME) fiber in the queen’s headspace. A blank was first obtained by exposing a fiber in the glass sampling chamber for 15 minutes. The queen was then transferred to the chamber and a new fiber exposed for a 15 minute sampling period to pick up a sample of the pheromones being emitted by the queen. Fibers were then analyzed using gas chromatography/mass spectroscopy (GC/MS) to separate the pheromone sample into its individual components and determine the relative abundance of each compound in that sample. Samples were analyzed on a per swarm basis to distinguish compounds belonging to the queen from ones naturally present in the sampling environment. Peaks belonging to the queen were integrated and a sum calculated for each sample to give a total integration value for each sample representing the relative amount of pheromone being emitted by the queen. A sum of the peaks belonging to the queen was also determined to represent the total number of discrete pheromonal compounds being released by the queen. Spearman correlations were used to evaluate relationships between the activity level and pheromone production of the queen. See table 3 for correlation coefficients, p values, and sample sizes. **A)** The activity level of the queen fails to correlate with the amount of pheromone she emits. **B)** The activity level of the queen fails to correlate with the number of pheromonal compounds she emits.
Figure 18. The relative amount of pheromone emitted by the queen positively correlates with the number of pheromonal compounds she produces. Pheromone profiling was executed via exposure of a solid phase micro-extraction (SPME) fiber in the queen’s headspace. A blank was first obtained by exposing a fiber in the glass sampling chamber for 15 minutes. The queen was then transferred to the chamber and a new fiber exposed for a 15 minute sampling period to pick up a sample of the pheromones being emitted by the queen. Fibers were then analyzed using gas chromatography/mass spectroscopy (GC/MS) to separate the pheromone sample into its individual components and determine the relative abundance of each compound in that sample. Samples were analyzed on a per swarm basis to distinguish compounds belonging to the queen from ones naturally present in the sampling environment. Peaks belonging to the queen were integrated and a sum calculated for each sample to give a total integration value for each sample representing the relative amount of pheromone being emitted by the queen. A sum of the peaks belonging to the queen was also determined to represent the total number of discrete pheromonal compounds being released by the queen. See table 3 for correlation coefficients, p values, and sample sizes.
Figure 19. The relationships between the queen’s pheromone production, the queen’s thoracic temperature, and the surface temperature of the swarm. Pheromone profiling was executed via exposure of a solid phase micro-extraction (SPME) fiber in the queen’s headspace. A blank was first obtained by exposing a fiber in the glass sampling chamber for 15 minutes. The queen was then transferred to the chamber and a new fiber exposed for a 15 minute sampling period to pick up a sample of the pheromones being emitted by the queen. Fibers were then analyzed using gas chromatography/mass spectroscopy (GC/MS) to separate the pheromone sample into its individual components and determine the relative abundance of each compound in that sample. Samples were analyzed on a per swarm basis to distinguish compounds belonging to the queen from ones naturally present in the sampling environment. Peaks belonging to the queen were integrated and a sum calculated for each sample to give a total integration value for each sample representing the relative amount of pheromone being emitted by the queen. A sum of the peaks belonging to the queen was also determined to represent the total number of discrete pheromonal compounds being released by the queen. Surface temperatures of queen and bivouac were measured via infrared photography. Utilizing specialized software, an area box was drawn over the area of interest on each image (queen’s thorax, entire bivouac surface, or bivouac surface in region located directly over the queen) and the mean temperature within that area determined. Spearman correlations were used to evaluate relationships between the queen’s pheromone production, the queen’s thoracic temperature, and the surface temperature of the swarm. See table 3 for correlation coefficients, p values, and sample sizes. A) The mean temperature across the entire bivouac surface positively correlates with the relative amount of pheromone emitted by the queen. B) The mean temperature across the entire bivouac surface positively correlates with the number of pheromonal compounds emitted by the queen. C) The mean temperature across the bivouac surface directly over the queen positively correlates with the amount of pheromone she emits. D) The mean temperature across the bivouac surface directly over the queen positively correlates with the number of pheromonal compounds she emits. E) The thoracic temperature of the queen positively correlates with the amount of pheromone she emits. F) The thoracic temperature of the queen fails to correlate with the number of pheromonal compounds she emits.
Discussion

In the current study, several relationships were observed between the metrics of the queen and the bivouac as the nest-site selection process reached a conclusion, scouts began to rouse quiescent workers to prepare for swarm liftoff, and, eventually, all bees flew toward a new home. Quiescent workers warmed their flight muscles as scouts begin signaling for an approaching liftoff, as has been observed in a previous study (Seeley and Tautz, 2001), and similar warming of the queen was observed in the present study as well. Furthermore, as queens got warmer, they also became more active, and they emitted a greater quantity of pheromones. Such increases were not found conclusively when all phases of the swarming process were compared, for reasons that are discussed below (i.e., variability among swarm replicates, methods that obscured differences in the present study, etc.). Previous research in our lab suggested that queens increase their pheromone production at the time of swarm liftoff relative to the amount of pheromones they emit before taking flight (Mattila et al.; unpublished data). A similar (but non-significant) trend was observed in this study. In general, these changes in queen physiology and behavior indicate that a swarming queen’s metrics change in a manner that is similar to workers in a bivouac (Seeley and Tautz, 2001; this study) as the swarm prepares to depart.

The results of this study also support the hypothesis that the workers within the bivouac played a key role in preparing the queen for liftoff. As she is often within the cluster, there is little opportunity for a queen to be directly aware of the progression of the nest-site selection
process that is occurring on the surface of the bivouac. This implies that other forms of input, coming either directly from the workers through such signals as piping or indirectly from them based on changes in the state of the swarm at large, help to warn her about an impending liftoff. The results of this study suggest that an increase in the temperature of the bivouac is a key form of input that helps prepare queens for swarm departure.

**Bivouac temperature strongly influences the behavior and physiology of queens as liftoff approaches**

The results of this study confirm the findings that bivouac temperatures increase as swarm liftoff nears and workers warm their thoracic muscles for flight (Seeley et al., 2003). Seeley et al. (2003) found that at swarm departure, workers had thoracic temperatures greater than 39.0°C, which is well above the 35°C threshold temperature that they must reach for flight. In the current study, infrared temperature readings were taken across entire bivouac surfaces as opposed to isolating individual worker thoraces, giving rise to a slightly lower mean temperature of 34.5°C (fig. 6B). This relatively lower temperature is likely a byproduct of taking the average temperature across the entire bivouac surface, which included the entire bodies of workers, with the peripheral body regions beyond the thorax being generally cooler in temperature.

In addition to a significant increase in the temperature of the swarm face, the queen’s thoracic temperature was also observed to rise significantly in the five minutes prior to swarm liftoff (fig. 6A). This increase in temperature could serve a dual purpose: 1) it would allow her flight muscles to be warm enough so that she too could take to the air and depart with the
workers in the swarm and 2) it could potentially aid in the volatization of her pheromone emissions at liftoff.

Results of this study strongly indicate that an increase in the temperature of the workers within the swarm cluster is involved with the queen’s increase in thoracic temperature approaching liftoff. The queen’s thoracic temperature positively correlated with the surface temperature of the entire bivouac (fig. 13B) as well as the surface temperature of the bivouac in the region directly over her (fig. 13C). This result suggests that the queen’s temperature rises with the temperature of the swarm cluster and that she could be utilizing the temperature of the bivouac of the workers surrounding her as a gauge for how close the swarm is to liftoff. With this information, she could then regulate her own body temperature accordingly so that her flight muscles are adequately warmed up for flight at the moment of departure.

Evidence of selective heating was also observed, wherein the location over the queen in the bivouac was maintained at a significantly higher temperature than the parts of the bivouac that were immediately adjacent (fig. 9). One possible cause for this temperature differential could be that the workers within the bivouac cluster in higher numbers directly over the queen’s chamber on the swarm stand. Higher numbers of bees in this region would result in a greater thickness of bees, increasing the insulative properties of the bivouac around the queen. While it was anecdotally observed that the center of the cluster fell above the queen’s chamber, the adjacent region chosen for testing of the selective-heating hypothesis was immediately next to the queen chamber. The bivouac on the swarm stand is usually thickest toward the top of the swarm stand where there is an extended ridge to which the bees attach
themselves, and the curtain of bees tapers off in thickness as you move downward (fig. 4). For this reason, the layer of workers directly over the queen should be of comparable thickness as the adjacent region used for comparison. Given that a significant difference was observed between these two areas, it is likely that the workers selectively maintained a higher temperature in this area to heat the queen as opposed to temperature differences occurring only because of a thicker insulative layer of bees over the queen’s location in the bivouac. This selective heating of the queen could aid her ability to keep her temperature slightly elevated, which could in turn assist in the volatization of her pheromones and her ability to maintain the cohesive nature of the bivouac prior to liftoff.

The temperature of the bivouac, both across the entire swarm face and in the region directly over the queen, was positively correlated with several queen metrics (i.e., queen thoracic temperature, activity level, total pheromone output and number of compounds produced; table 3; figs. 13B, 15B, 17A, 17B), which suggests that bivouac temperature plays a role in preparing queens for liftoff. While this study also examined a more direct signaling pathway as possible mechanisms of preparing her for swarm liftoff (i.e., the increase in piping signals that are produced by workers as liftoff approaches) this finding raises the possibility that queens are also getting indirect information about swarming status by assessing the temperature of the workers around her. Rather than active signaling through piping of the queen being the only component that could lend to her preparation, it is possible that a gradual increase in bivouac temperatures approaching swarm liftoff also cues modifications in her behavior and physiology.
Changes in pheromone production by queens during liftoff

Previous work in our lab indicated that queens increase their pheromone production at the time of swarm liftoff (Mattila et al.; unpublished data). However, the results of the current study did not find a significant change in either total pheromone emission or number of unique chemical components that queens produced in the moment of liftoff compared to pre-liftoff phases of the swarming process (fig. 12). These contrasting results are likely attributable to differences between studies in how queens were handled during pheromone sampling. Methods for queen transfer to the pheromone sampling chamber differed between the two studies, with the method in this study requiring more handling of the queens. This might have antagonized the queens and kept them more active during sampling at the start of the swarming process, significantly altering their pheromone production. This difference probably also explains why queen thoracic temperature was higher at the time of swarm set up than what it was after swarms had settled (i.e., it was statistically similar queen temperatures at swarm liftoff).

Other source of variability that might have affected the pheromone output results across time points includes the possibility that the solid phase micro-extraction (SPME) fibers that were used for sampling varied in their absorbency. A study by Niedziella et al. (2000) found that the absorbency of the specific type of SPME fibers utilized in this study can decrease over time with repeated usage. In support of this idea, several samples that yielded abnormally low peak abundances were traced back to two fibers which exhibited a reduced ability to absorb chemicals. These data points were removed from consideration for analysis purposes, but this
occurrence points to the fact that fiber absorbency was not controlled in this study. An internal standard might have better controlled for this variation and will likely be implemented in follow-up studies that are planned for summer 2012.

Alternatively, if the results of this study accurately reflect the pheromone production of the queen during the swarming process, it is possible that she increases her pheromone production from her in-colony, pre-swarming norm (as indicated by Mattila et al.; unpublished data) to the heightened levels that were observed in both years within the bivouac, but that she does not significantly alter her pheromone emission once the colony has entered the bivouac stage after departing from the colony. In the previous study, sampling was conducted at three time intervals: in the parental colony, at the time of bivouac set-up, and at the moment of liftoff. The current study provides the first insight into pheromone production between bivouac set-up and swarm departure. Although many metrics of queens and bivouacking workers increased in the hour or minutes leading up to swarm liftoff, if the results of this study accurately portray the queen’s pheromone output, it is possible that she increases her pheromone output as the swarm leaves the colony, but maintains relatively constant levels of pheromone production through the remainder of the swarming process.

On the whole, despite the discrepancies observed between the current study and the findings of Mattila, et al. (unpublished data), it is important to note that the findings of both studies share similar trends. The same general upward trend in pheromone production was observed between bivouac set-up and swarm liftoff, with high levels of variation preventing these differences from being significant in the current study (fig. 12). In addition to this, several
significant correlations were found between the queen’s pheromone output and metrics of the
swarm (table 3), suggesting that they at least, in part, accurately reflect the nature of the
queen’s pheromone production during the swarming process and should not be discounted
from consideration.

The role of piping signals in preparing queens for swarm liftoff

Piping signals significantly increased approaching swarm liftoff, suggesting an important
role in warning inactive workers of the impending departure. The results of this study agree
with those of Seeley and Tautz (2001) who found that piping signals that were produced by
scouts increased in frequency in the hour leading up to swarm departure, with the highest
frequencies observed in the moments before swarm liftoff. Piping signals also showed a strong
tendency to increase as the surface temperature of the entire bivouac increased ($p = 0.06$; table
3; fig. 14A), suggesting that this signal has an important role to play in warning quiescent
workers to warm up their flight muscles so that they are able to go airborne at liftoff.

In contrast to the clear connection between piping signals and worker preparation for
swarm departure, results concerning relationships between the production of piping signals
and metrics of queens were not as straightforward. Piping signals positively correlated with the
total pheromone production of the queen (table 3; fig. 16A), but failed to significantly correlate
with her thoracic temperature (table 3; fig. 14C), activity level (table 3; fig. 15A), or number of
discrete chemical components present in her pheromone samples (table 3; fig. 16B).
Considered together, this study was not able to conclusively establish a clear relationship
between the piping signals that are produced by workers and all of changes that occur in queen
physiology and behavior as she prepares for swarm departure. Previously published literature, however, suggests that this uncertainty should be further pursued.

A study by Pierce et al. (2006) suggests that workers increasingly pipe the queen in the days prior to swarm issue from the original colony, as well as during the 2 to 4 hours that lead up to swarm departure from the bivouac toward a new nest site. Queen are subjected to much higher rates of piping in the colony (8-19 signals per minute) as opposed to within the bivouac (2-6 signals per minute) in the hours leading up to departure (Pierce et al., 2006). Rates of piping of the queen were 5-30 times lower within the cluster than within the colony. This result suggests the possibility that piping of the queen by workers within the colony is more important than it is within the bivouac. Here, we propose that, while piping of the queen is the main form of signaling that warns her of an impending swarm issue from the original nest, within the bivouac it is a combination of increases in the temperature of the bivouac and piping signals (in some ways) that are used together to prepare her for departure.

Although our study yielded no significant correlation between piping signals and most queen metrics (table 3), the study by Pierce et al. (2006) also demonstrated that queens are selectively targeted with piping signals during the bivouac phase of the swarming process. Indeed, scouts that had visited potential nest sites sought out the queen within the cluster and were responsible for a mean of 30% of all piping signals that were performed on her. Given the specificity of queen targeting and the investment of energy that workers are making in providing queens with information about the swarm’s state, it seems likely that piping does play some role in preparing the queen for liftoff. One explanation for our lack of significant
correlations between piping and queen metrics could be the assignment of 60 piping signals per
minute to time points where piping was continuous near liftoff. These data points served as a
minimum estimate of piping intensity, but may have been gross underestimations of actual
rates of piping that concealed the correlative relationship of piping to other queen metrics. We
are currently investigating alternative methods of interpreting our raw data to more accurately
represent piping intensity at liftoff in comparison to other phases in the swarming process.

A second possible explanation for our lack of significant correlations between piping
signals and metrics of the queen could be that the piping signal is largely a contact signal and
the cage that separated the queen from the cluster might have prevented the successful
transmission of these signals. Pierce et al. (2006) kept their queens in a cage that workers could
enter and exit (thus, they could pipe her freely), whereas workers in our study had to pipe the
queen through a mesh screen (which they are known to do, but this barrier still makes her less
accessible to them). Interestingly, even though workers had more limited physical contact with
queens, it is noteworthy that the queen’s flight muscles were still warm enough for flight at the
moment of swarm departure, suggesting that other factors (such as an increase in the
temperature of the bivouac approaching liftoff) are probably key for her preparation.

The influence of the queen’s activity level in her preparation for swarm liftoff

The relationships between the activity level of queens and other aspects of their
behavior and physiology as they prepare for swarm departure yielded mixed results. The
activity level of workers is known to increase approaching swarm liftoff (Rittschof, Seeley,
2008) and we hypothesized that a similar increase might be observed in the queen. We further
supposed that an increase in a queen’s activity level could have been linked to an increase in her metabolic activity and temperature, which could ultimately enhance the quality and quantity of her pheromone emissions. In contrast to our expectations, the queen’s activity level was not observed to significantly increase approaching swarm liftoff (fig. 11), although the trend did approach the level of significance (p = 0.10) and it is likely that the potential significance of this trend would be confirmed with more swarm replicates. However, in line with our expectations, activity level was positively correlated with the temperatures of the bivouac (table 3; 15B), the bivouac in the area over the queen (table 3; fig. 15C), and the thoracic temperature of the queen (table 3; fig. 15D). It was also marginally associated with total pheromone output (p = 0.07; table 3; fig 17A) and the number of discrete compounds that queens produced (p = 0.09; table 3; fig. 17B). Increased activity by a queen is likely is a key indicator that she is receiving signals that prepare her for the swarm’s impending departure, but we need to gather more data before definitive conclusions about how activity influences pheromone production can be made.

**Directions for future work**

While this study sheds light on how swarming workers and queens interact to prepare the swarm collective for a risky airborne flight to a new home—one that only a few members of the swarm have ever visited—much remains to be learned about how the all-important queen stays attuned to the natural progression of this process. No study before this one has revealed that queens heat up as workers do in preparation for flight, or that workers selectively heat queens at all points in the swarming process, perhaps facilitating her production of
pheromones that help keep the bivouac cohesive. This study also points to the fascinating possibility that the queen uses the temperature of the bivouac as a method to gauge the progression of the nest-site selection process so that she knows when to prepare herself for liftoff. While this study yielded these interesting findings, correlations cannot be equated with causation and it is therefore our intention to conduct manipulative field experiments during the summer of 2012 to determine exactly how the queen’s exposure to bivouacking workers influences her preparation for liftoff. We will do this by cutting off the queen’s access to workers within the bivouac and evaluating whether, without this input, she fails to prepare for swarm departure.

Our preliminary experimental design will place two queens within a single bivouac (neither being a queen native to the colony from which the swarm was created). One queen will be accessible to workers within the swarm, with the expectation that we will observe the same changes in her physiology and behavior as liftoff approaches, while a second queen will be isolated from the bivouac by a double-screen enclosure, so that workers will be able to sense her pheromonal presence, but they will not be able to contact her to pass piping signals that may warn her of liftoff. Because this second queen needs workers to feed and care for her (she would perish if isolated from her retinue), we will provide her with a separate retinue of naïve workers who can access her from the back of the swarm stand, where they too will be isolated from the bivouac and its preparations for swarm liftoff. The metrics of the two queens (one subjected to workers who are participating in the nest-site selection process and the other isolated from signals related to this process) will be compared to determine whether physical
isolation from the bivouac prevents the queen from heating up and producing more pheromones as the swarm prepares for liftoff.

In addition to permitting us to establish the nature of the signals that are necessary to prepare a queen for liftoff, this follow-up study will grant the opportunity to repeat our pheromone sampling with more refined methodology so that we can definitively characterize changes in the queen’s pheromone production as liftoff approaches. Part of the discrepancy between the SPME data from this study and the previous year (Mattila et al.; unpublished data) likely stems from excessive handling of queens during her initial transfer to the sampling chamber from the screened swarm-prep cage, as well as variation in the absorbency of some of our SPME fibers. To avoid these difficulties going forward, we plan to expose SPME fibers to the headspace of each queen while she is still in her cage on the swarm stand, which will avoid the disturbance of removing her from the stand and handling her prior to the conclusion of the swarming process (something that did not occur in the first study). One downside of this approach is that we anticipate that sampling directly from the swarm stand will heighten the possibility of environmental contaminants being absorbed by the fiber (a caged queen adjacent to bivouacking workers presents a more complex chemical environment than a queen held in a previously cleaned queen chamber). To limit the complication of differentiating environmental and queen compounds, we will examine only the quantities for the known major chemical components of queen mandibular pheromone. Furthermore, we will use an internal standard for each sample to control for variation in absorbency between SPME fibers. On the whole, this follow-up study should enable us to solidify our understanding of the changes in queen metrics
approaching swarm liftoff and to establish which of these factors are most critical in preparing queens for the precarious journey to a new nest site.
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