12-2010

Lack of Rhythmicity in the Honey Bee Queen: An Investigation of Temporal Behavioral Patterns in *Apis mellifera ligustica*.

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A thesis

presented to

the faculty of the Department of Biological Sciences

East Tennessee State University

In partial fulfillment

of the requirements for the degree

Master of Science in Biology

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by

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December 2010

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Keywords: *Apis mellifera*, honeybee, queen behavior, circadian rhythms

ABSTRACT

Lack of Rhythmicity in the Honey Bee Queen:

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Little is known about the behavioral patterns of honey bee queens. To determine if mated honey bee queens possess diel rhythmicity in behavior, we observed them in glass-sided observation hives using three types of observation regimes: focal studies consisting of 2-hour and 24-hour continuous observations as well as scan-sampling of multiple queens. All behaviors (active: walking, inspecting, egg-laying, begging for food, feeding, and grooming self; inactive: standing) occurred at all times of day and night, but no queen showed consistent diel rhythmicity in any of the individual behaviors. There were no consistent diel differences in active *versus* inactive behaviors or the number of bees in the queen's retinue. This arrhythmicity was unchanged despite daily changes in both light and temperature levels. The arrhythmic behavior observed by most of the honey bee queens inside the colony appears to be similar to that exhibited by worker bees before they initiate foraging behavior.

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CHAPTER 1

INTRODUCTION

Honey bees (*Apis mellifera*) are eusocial insects co-existing in a colony and functioning as a single entity (organism). The hive is made up of one queen, several haploid male drones, and up to 80,000 diploid female workers (Butler 1949). The workers progress through different duties inside the hive such as hive maintenance (repair and buildup of comb, cleaning cells, etc.), brood care (tending eggs and feeding larvae), queen care (grooming and feeding the queen), receiving and storing nectar, and packing pollen before they leave the nest to perform outside duties such as foraging (collecting pollen, water, and nectar) (Seeley 1982, 1995; Robinson, 1992).

The queen and the drones function solely as reproductive entities. Drones continue to develop after they emerge from the cell, reaching sexual maturity at approximately 12 days (Winston 1987). After reaching sexual maturity, the drones begin flying to a drone congregation site, in hopes of mating with a queen from another hive. The exact specifications for the drone congregation site are not well known, but the drones do base their flights on time of day and temperature (Rowell *et al.* 1986), suggesting an endogenous clock mechanism. The queen's primary focus is to lay eggs. She usually lays on average less than 1,500 eggs per day (Winston 1987), but that depends on temperature (Dunham 1930), pollen stores (Schmickl *et. al.* 2003), and seasonal factors (Sasaki and Obara 2001).

The most commonly studied caste of bee is the worker whose circadian rhythms have been studied extensively (Moore and Rankin 1985; Moore *et al.* 1989; Moritz and Sakofski 1991; Moore *et al.* 1998; Moore 2001; Bloch and Robinson 2001). It has also been shown that

the in-hive duties such as brood care lack a natural rhythm (arrhythmic) and happen 24 hours a day (Moore *et al.* 1998; Bloch and Robinson 2001). The circadian rhythms of the queen have not been studied as extensively as the rest of the hive, and this will be the main focus of the study.

Even though they potentially share the same parents, honeybee queens differ developmentally, morphologically, and sexually from their nest mates and have a very distinct role and importance to the hive. It is known that their behavior differs from that of their female nest mates (drones will not be addressed further in this paper) in that they do not progress through several different behavioral stages with age but instead remain in one phase post-mating (reproduction). Worker bees have been shown to develop a circadian rhythm as they age (Moore *et al.* 1998; Bloch and Robinson 2001) and move from in-hive duties to outside duties. They do not have a circadian rhythm until the final stage in their development. Because queen bees have a different developmental profile and develop with a different behavioral suite from their hive mates, we want to see if they acquire a circadian rhythm or stay arrhythmic. In order to show rhythmicity in a queen (or lack thereof), her minute-by-minute activities, night and day activities, and seasonal activities have to be observed and documented.

Questions of the Research

***Question 1*:** How does the queen organize her time on a minute-by-minute basis?

Hypothesis 1: The queen's actions are random; therefore, no pattern will emerge in her behavior.

The queen has been observed inspecting cells, laying eggs, begging for food, grooming self, feeding, and walking, all of which are classified as 'activity'; she has also been observed standing still, which is considered 'inactive'. It is possible that she spends her time randomly

divided between activity and inactivity such that no pattern emerges, and no predictions can be made as to whether she will be active or inactive at any given time.

Hypothesis 2: The queen's actions are based on her location in the hive.

The hive is usually composed of empty cells, cells containing eggs or larvae (brood cells), and cells containing honey, nectar, or pollen. The queen may travel over all of these cells, but do they play a role in her activities? Does an inspection of an empty cell trigger the need to fill the empty cell with an egg? If the queen discovers an area of unavailable cells, does she then take a break or keep searching until she discovers some more empty cells? If substrate plays a role in her behavior, then she should continue to lay eggs nonstop as long as cells are available near her.

Hypothesis 3: The queen's actions are based solely on internal programming.

It is also possible that the queen's actions are neither random nor substrate-based, but occur based on a preprogrammed internal mechanism. She could be predisposed to a certain behavior for a certain period of time followed by another behavior for another length of time. For example, she could inspect cells and lay eggs for *x* minutes, then switch to resting for *y* minutes, and after that, resume cell inspection and egg laying for *x* minutes, etc.

Question 2: How does the queen partition her time from night to day?

Hypothesis 1: The queen's bouts of activity occur more frequently during daylight hours.

Whether or not a minute-by-minute pattern exists, we may discover that she has a rhythm that coincides with day and night. Most animals show some sort of rhythmic activity that occurs

daily (diurnal), nightly (nocturnal), or at dusk and dawn (crepuscular). The queen's activities may be similar to those of the diurnal foragers who are more active during the day and rest at night (Moore 2001).

Hypothesis 2: The queen's activity will increase during certain phases of the day not corresponding to diurnal, nocturnal, or crepuscular, such as ultradian or infradian.

The queen's activity in relation to her resting periods may increase during certain hours throughout the day that cannot be classified as diurnal, nocturnal, or crepuscular but does occur at approximately the same time each day. For example, the queen may become more active mid afternoon, and her activity may continue until after sundown. Her shift of activity would cover six hours that includes daylight, dusk, and night. Therefore it could not be classified into just one category, but it would still be rhythmic because it occurs in the same time frame every day.

Hypothesis 3: The queen's activity is arrhythmic in relation to night and day.

The queen's activity may not significantly increase as compared to resting at any point during the day or night. Her bursts of rest and activity might be randomly disbursed throughout the 24-hour period. This behavior would follow that of newly emerged arrhythmic bees. Moore *et al.* (1998) found that young bees (less than 20 days old) have no circadian rhythm, but they do develop a circadian rhythm when they become foragers (approximately 20 days old) and venture outside the hive. The queen only flies from the hive to mate, so her lack of rhythm may mimic that of pre-foragers.

***Question 3*:** How does the queen's behavior change annually?

To our knowledge a colony (and more specifically, the queen) has not been observed for an entire 12-month period. Free *et al.* (1992) observed colonies from October 1958 to March 1959 for 30 minutes per day for 5 days per week (time of day not specified) and then again from October 1980 to February 1981 for approximately 65 minutes for an unknown number of days. They reported no diel periodicity seen in the queen, but they only looked at distance traveled during a 30-minute period of time.

Hypothesis 1: The queen's activity changes throughout the year based on photoperiod.

The queen may have an internal circannual clock that helps regulate egg laying according to season. Typically, a circannual clock is what helps an animal know when to hibernate based on photoperiod. As winter approaches the daylight hours get shorter. This could be a cue to slow down egg laying. The colony size shrinks in the winter because forage is limited, and the bees must live on the stored honey and pollen. The queen would not need to produce many eggs because food resources are limited, and overproduction of eggs could lead to a colony's demise if it were allowed to continue. For the benefit of the colony, the queen should not stop laying eggs completely. If she were to perish, the workers would still have a constant supply of eggs available to them from which they could create a new queen. In the spring, the photoperiod gets longer, which allows the bees more daylight hours to gather nectar and pollen. The longer daylight hours may be a cue to let the queen know when to boost egg laying. In the spring the colony needs to replenish itself and prepare for foraging; therefore, egg laying must increase.

Hypothesis 2: The queen's activity would change in relation to the photoperiod, and any pattern found in Question 1 or Question 2 would change also.

This hypothesis is based on the findings from Question 1 and/or Question 2, which are not yet known. For example, if we find that her activity in spring follows a diurnal rhythm, this rhythmicity will be less pronounced in winter or may disappear completely.

As stated earlier, workers develop a circadian rhythm over time in their natural environment. Toma *et. al.* (2000) took pupae from a hive in the field and allowed them to emerge in the incubator in the lab. The newly emerged bees were then transferred to an environmental chamber where locomotor activity was monitored constantly. The bees had never been exposed to other bees and were kept in constant conditions (darkness, 26^{o}C) throughout the experiment. The experimental bees began life with no rhythm but developed a distinct circadian rhythm exactly like their counterparts in the wild. If the queen shows any sign of rhythmicity, we will be able to confirm our findings via this chamber in a similar experiment.

CHAPTER 2

ABSENCE OF CONSISTENT DIEL RHYTHMICITY IN MATED HONEY BEE QUEEN BEHAVIOR

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Abstract

Relatively little is known about the temporal control of behavior of honey bee queens under natural conditions. To determine if mated honey bee queens possess diel rhythmicity in behavior, we observed them in glass-sided observation hives employing two focal studies involving continuous observations of individual queens as well as a scan-sampling study of multiple queens. In all cases all behaviors were observed at all times of the day and night. In four of the five queens examined in focal studies, there were no consistent occurrences of diel periodicity for any of the individual behaviors. A more encompassing measure for periodicity in which the behaviors were characterized as active (walking, inspecting, egg-laying, begging for food, feeding, and grooming self) or inactive (standing) also failed to reveal consistent diel rhythmicity. Furthermore, there were no consistent diel differences in the number of workers in the queen's retinue. Behavioral arrhythmicity persisted across seasons and despite daily changes in both light and temperature levels. Both day and night levels of behavioral activity were correlated with daytime but not with nighttime, ambient temperatures. The behavior of the one exceptional queen was not consistent: diurnal activity patterns were present during two 24-h observation sessions but arrhythmicity during another. Based on the behavior observed by all but one of the queens examined in this work, the arrhythmic behavior by the mated honey bee queen inside the colony appears to be similar to that exhibited by worker bees before they approach the age of onset of foraging behavior.

Keywords: *Apis mellifera*; Queen; Behavior; Circadian rhythm; Social insects

1. Introduction

One of the more remarkable features of the circadian system controlling behavior in European honey bees (*Apis mellifera*) is its plasticity. Typically, honey bee workers perform an age-related sequence of in-hive tasks (an integral component of the division of labor) from emergence until they assume foraging duties at about 21 days of age (Lindauer, 1952; Sakagami, 1953; Seeley, 1982; Robinson, 1992). Newly emerged workers are arrhythmic whether individually isolated under constant conditions (Spangler, 1972; Toma et al., 2000) or housed in observation colonies under natural conditions (Moore et al., 1998). In the colony young workers perform their age-associated in-hive tasks around-the-clock but develop rhythmicity in advance of becoming foragers (Moore et al., 1998). This rhythmicity is diurnal: workers exhibit higher levels of resting behavior at night relative to the day. Behavioral rhythmicity is well developed by the time workers attain foraging status. Although different foraging groups may be active at different times of day (Körner, 1939; von Frisch, 1940; Moore et al., 1989), foragers display strong diurnal rhythmicity by resting in the hive at night, even displaying sleep-like characteristics (Kaiser and Steiner-Kaiser, 1983; Kaiser, 1988; Sauer et al., 2003; Klein et al., 2008). The ontogeny of rhythmicity also is evident in individually isolated workers. Initially arrhythmic (Spangler, 1972; Toma et al., 2000), young worker bees develop free-running activity cycles under constant dark and constant temperature conditions after several days. Isolated foragers show strong circadian rhythms of locomotor activity under constant conditions (Spangler, 1972; Moore and Rankin, 1985; Toma et al., 2000; Bloch et al., 2006; Shemesh et al., 2007) and exhibit diurnal entrainment patterns to a broad spectrum of different light-dark (LD) and temperature cycles (Moore and Rankin, 1993). Similar patterns are seen in Japanese honey bee (*Apis cerana japonica*) foragers (Fuchikawa and Shimizu, 2007a,b).

The ontogeny of behavioral rhythmicity in honey bees appears to be adapted to the needs of the colony. Most in-hive tasks are performed around-the-clock. One example is brood care behavior, conducted primarily by "nurse bees" (approximately 5-15 days of age). It is thought that the unremitting day and night attention received by the brood from nurses (Crailsheim et al., 1996; Moore et al., 1998; Shemesh et al., 2007) may contribute to colony efficiency (Bloch, 2009). Honey bee foragers in contrast exhibit robust diurnal circadian rhythms. Foragers rely on a continuously consulted circadian clock to navigate through the environment using a time-compensated sun compass (von Frisch, 1967) and to schedule their foraging visits within optimal time-windows for collection of nectar and pollen from different species of flowers (reviewed in Moore, 2001).

The expression of behavioral rhythmicity of both nurses and foragers is modifiable. When arrhythmic nurses are removed from a colony that is maintained under a LD cycle and transferred to individual chambers under constant laboratory conditions, they exhibit free-running circadian rhythms of locomotor activity with the active phase during subjective day (Shemesh et al., 2007). Forager honey bees can be induced by removing most of the nurses from the colony to revert to nursing behavior (Huang and Robinson, 1996). Most reverted nurses lose their rhythmicity, performing brood care behavior around-the-clock (Bloch and Robinson, 2001; Bloch et al., 2001). The age of onset of rhythmicity in locomotor activity is earlier for newly emerged workers that are housed with about 30 foragers than for those housed with the same number of young bees (Meshi and Bloch, 2007), thus revealing a social influence on the ontogeny of behavioral rhythmicity. The circadian systems of worker bees also may be sensitive to seasonal effects: free-running periods of locomotor activity in individual foragers and nurses increased from spring to summer (Bloch et al., 2006).

Compared to workers a relative paucity of information exists concerning the circadian system controlling behavior in honey bee queens. Presumably, virgin queens possess a working circadian clock so that they can schedule their nuptial flights at the appropriate time of day (Koeniger and Koeniger, 2000) and navigate to and from drone congregation areas. A number of physiological and behavioral changes are associated with the transition from virgin to egg-laying status. For instance, virgin queens are initially photonegative but become phototactic in anticipation of their nuptial flights (Berthold and Benton, 1970). Once egg-laying is initiated, however, honey bee queens once again become photonegative and refrain from further mating flights for the remainder of their lives (Berthold and Benton, 1970; Winston, 1987; Kocher et al., 2008). The recently mated queen also exhibits growth and maturation of the ovaries (Patricio and Cruz-Landim, 2002), radical changes in pheromone profile (Plettner et al., 1997; Keeling et al., 2003), an increase in neuropil volume and a decrease in the volume of Kenyon cell somata in the mushroom bodies (Fahrbach et al., 1995), and a decline in dopamine levels (Harano et al., 2005) leading to a reduction in locomotor activity (Harano et al., 2008). Genomic analyses are beginning to uncover the molecular mechanisms underlying these changes (Richard et al., 2007; Kocher et al., 2008). In accord with worker honey bees exhibiting around-the-clock performance of in-hive tasks (Moore et al., 1998), it would be reasonable to expect the queen to show similar arrhythmicity once she assumes egg-laying status.

With respect to behavior mated queens in observation colonies showed no evidence of diel periodicity in the number of eggs laid, distance traveled, or the number of attendants in her retinue (Free et al., 1992). However, in a recent study of individually isolated honey bee queens (Harano et al., 2007), the locomotor activity of all virgin queens and most mated queens exhibited free-running circadian rhythms under constant conditions. The apparent absence of

rhythmicity in mated queens under natural conditions and its presence under constant conditions in isolation is reminiscent of the behavioral plasticity seen in nurse bees (Shemesh et al., 2007). Plasticity in behavioral rhythmicity has been well described for queens of several different ant species – virgin queens exhibit circadian locomotor activity but become arrhythmic when laying eggs (McCluskey, 1992; Sharma et al., 2004a). Furthermore, in the ant *Camponotus compressus*, queens resumed rhythmicity after termination of the egg-laying phase (Sharma et al., 2004b).

It is difficult to discern the presence or absence of diel behavioral rhythmicity without knowing the full range of behaviors possible within the organism's environment. The situation is relatively simple for studies of locomotor activity under laboratory conditions: the organism simply is active or it is not. In contrast the situation may be much more complex under natural conditions in which the organism may exhibit an array of different overt behaviors, interact with other individuals, and respond to environmental changes. One plausible scenario is that some behaviors are rhythmic while others are not. Alternatively, rhythmicity may not appear at the level of individual behaviors (i.e., walking, mating, oviposition, etc.) but nevertheless emerge if the different identifiable behaviors are viewed in terms of 'active' and 'inactive' groups. For instance, worker honey bees perform a variety of in-hive tasks as they progress through their age-polyethism schedule (Lindauer, 1952; Sakagami, 1953; Seeley, 1982; Robinson, 1992). None of these 'active' behavioral tasks appear to be rhythmic (Moore et al., 1998). However, as workers approach foraging status, they take more rest breaks (typically only a few minutes per occurrence) at night relative to the daytime hours, thus revealing a diurnal rhythmicity that does not sacrifice around-the-clock task performance (Moore et al., 1998).

In the present study we examine the entire repertoire of the queen's behavior within glass-sided observation colonies using two approaches – focal observations of individual queens and scan-sampled observations using multiple queens. In both cases the queens are not isolated but observed within the milieu of working colonies. Our primary goal is to determine the presence or absence of diel rhythmicity based on day and night measures of each of the queen's overt behaviors as well as the manner in which she integrates her presumptive resting behavior with her more obviously active tasks. Our observations build upon those of Free et al. (1992) who studied a somewhat limited selection from the queen's behavioral repertoire.

2. Materials and methods

2.1. General procedures

Behavioral observations were made on naturally mated queens (*Apis mellifera ligustica*) within working colonies (*i.e.*, unrestricted access to the natural environment) kept in glass-sided observation hives. The observation hives were situated at five different sites, all within or near Johnson City, Tennessee. Ambient temperature was measured with digital thermometers in the immediate vicinity of the colony at mid-hive level. Natural day-night cycles were noted using civil twilight to determine sunrise and sunset times. In all cases the colony was exposed to daily variations in light levels through the hive entrance. The observation rooms were not illuminated.

2.2. Behavioral observations

Three different studies were performed. The first involved a scan sampling strategy involving multiple queens designed to determine, in general, the presence or absence of different queen behaviors at all times of the day and night. The second study, providing a higher temporal

resolution of behavioral performance than the scan-sampling approach, involved continuous monitoring of individual queen activity: queen bees were monitored during four specific observation sessions (two during the day and two at night) each 120 minutes long for three consecutive days. These three-day observation sets were conducted at monthly intervals, encompassing the transition from late summer to mid-winter for one queen and late winter to early summer for two other queens. In the third study, serving to corroborate and extend the previous studies, three different queens were monitored during continuous 24-h observation sessions during the summer. In all the studies the queens were free to move to either side of the colony.

For the continuous monitoring (focal) studies, the queen's behaviors were recorded manually during each observation session. Observers positioned themselves at eye-level with the queen. Using quickly executed, hand-written symbols to record each behavior on data sheets previously set up in one-minute sections, the observers achieved real-time monitoring of the queen's activities. Furthermore, queen behaviors proved to be highly stereotyped and easily distinguished from one another (Table 1). One behavior (grooming self) was a complex of several different motor patterns including cleaning of the eyes, antennae, abdomen, legs, and proboscis. Each instance of inspecting (inserting head in cell) and egg-laying (inserting abdomen in cell) was recorded as a separate occurrence. It is important to note here that for focal study 1 three behaviors (begging for food, feeding, and grooming self) were treated separately from standing behavior despite the fact that all three occurred when the queen was stationary. Standing behavior, therefore, was defined as being stationary but not also performing any other action. For focal study 2 the recording process was streamlined: begging for food, feeding, grooming self, and standing were all designated as 'stationary' behaviors.

Table 1 Description of behaviors exhibited by queen honey bees.

Behavior	Description
Standing	Motionless on comb, frame, or glass; not begging for food, feeding, or grooming self
Walking	Locomotion across comb, frame, or glass
Inspecting	Placing head inside a cell
Egg-laying	Placing abdomen deep inside a cell; usually results in egg deposition
Feeding	Using proboscis to retrieve food from worker bee's mouthparts
Begging for food	Extending proboscis toward another bee; no contact is made
Grooming self	Rubbing proboscis, eyes, antennae, legs, or abdomen with legs

For the purposes of the focal queen studies it was required that every behavioral transition as well as the duration and time of day of occurrence of every behavior be recorded. To ensure that no behaviors were missed, but nevertheless maintaining reasonable temporal resolution, we estimated the durations of individual behaviors if more than one was performed in any given minute. For example, if one instance of walking, two instances of inspecting, and two instances of egg-laying were all performed within the same minute, then each occurrence of behavior was estimated to have occupied one-fifth of that particular minute.

In the scan sampling study each scan sample consisted of locating the queen and noting only the *first* behavior seen. Walking, stationary, inspecting, and egg-laying behaviors were monitored. Feeding, begging for food, and grooming self behaviors were relatively rare and, therefore, combined with standing behavior as 'stationary,' as in focal study 2.

2.3. Scan sampling: multiple queen study

Three trials were conducted: August 25-September 2 (7 colonies); September 8-13 (6 colonies); and October 3-7, 2008 (5 colonies). Each trial consisted of scan sampling (Altmann, 1974) queen behavior in four-frame observation hives kept at three different study sites. All of the observations were made by two observers (JJ and CG) with JJ accounting for 50%, 60%, and 70% of the observations in the three successive trials. Discrimination among the four monitored behaviors (stationary, walking, inspecting, and egg-laying) proved to be quite simple for both observers. Brief observation sessions prior to the study revealed no differences in interpretation

of these behaviors. The queens at each study site were naturally mated and obtained from different commercial sources (Table 2). Sites A, B, and C were in a garage, a large barn, and a small barn, respectively. All colonies had free access to the environment but also were provided with 1M sucrose and pollen continuously at the hive entrance. The same colonies were used in each trial but colony A1 was removed from trials 2 and 3 because of intense nectar robbing from stronger colonies. Colony B2 was not included in trial 3 because a second queen emerged just prior to the onset of the trial. The queen from colony A2 was removed from her original colony but re-established with workers from other, unrelated colonies and used in trial 3. This particular colony, quite small in population size, was housed at study site C rather than site A to protect it from the influence of nectar robbing. Colony size was estimated to the nearest 100 bees by counting the bees from 10 randomly chosen grid squares (drawn on the glass) on each side of the hive and making the appropriate extrapolations. All of the queens were obtained from suppliers (Table 2: Gardner's Apiaries, Baxley, GA; Rossman Apiaries, Moultrie, GA; Wilbanks Apiaries, Claxton, GA) and installed in the colonies during July 2008.

Table 2 Study site, commercial source for queen, and estimated population (nearest 100 bees) of each colony for all three trials in multiple queen study.

Colony	Commercial source	Study site	Estimated colony population		
			Trial 1	Trial 2	Trial 3
A1	Gardner's	A	900		
A2	Rossman	A (trials 1 and 2), C (trial 3)	2600	2200	100
B1	Rossman	B	5600	2500	5300
B2	Wilbanks	B	2000	2600	
B3	Gardner's	B	6800	6500	7600
C1	Gardner's	C	7700	6300	8000
C2	Wilbanks	C	5300	4500	2200

Although the hives were sheltered from direct sunlight at each study site, they were otherwise exposed to natural day-night oscillations in temperature and light levels. Observations (using a red LED headlamp during the night and a small flashlight during the day) were taken every 30 minutes during 3-hour sessions (for all of the colonies present at each

particular study site) over the course of 9, 6, and 5 days, respectively, for the three successive trials. The observer was allotted 10 minutes per hive to find the queen and note her first behavior upon discovery. If the queen was not located within 10 minutes, the observer moved on to the next colony. All of the queens were located within the 30-min scan sample, with only three exceptions (twice in trial 2 and once in trial 3). A minimum waiting period of 10 minutes was required between consecutive observations of the same queen. Each queen was sampled around-the-clock, but with no more than two 3-hour sessions on any given day. If two sampling sessions were scheduled on the same day at the same study site, they were separated by at least 9 hours. Emerging from this sampling scheme was a composite view of queen behavior over the entire day-night cycle, with a resolution of 0.5 h.

2.4. Focal study 1: four 2-h observation sessions per day

Three different, unrelated queens (numbered 1, 2, and 3) and their colonies were monitored. Two colonies (1 and 2) were housed within three-frame observation hives located within a walled-off section of a laboratory, three stories above ground level, and exposed to natural LD conditions (and therefore bright light during the day) at the hive entrance but not exposed to artificial light in the observation room (light levels were not at absolute darkness, but sensors pointed away from the glass sides of the hive always measured 0 lux). The third queen was kept in a four-frame observation colony housed in an observation shed under similar lighting conditions. Queen 1 was approximately one month of age and queens 2 and 3 approximately 10 months of age at the beginning of their respective observations. Each queen was monitored during four separate, 120-minute observation sessions each day for three consecutive days using dim red light, invisible to the bees (Menzel and Blakers, 1976). Two sessions were scheduled at night and two during the day. Based on local sunrise and sunset times, the early-day observation

session was centered between sunrise time and the midpoint time of the current natural photophase. Similarly, the late-day session was centered between the midpoint of the photophase and time of sunset. The early- and late-night observation sessions were scheduled at a 12-h phase difference from the early-day and late-day sessions, respectively. This scheduling resulted in the early-night observation sessions beginning approximately 2 hours after sunset and the late night sessions 4 hours before sunrise. The early day sessions began about 2 hours after sunrise and the late-day sessions 4 hours before sunset. The 3-day observation sets were conducted once per month at monthly intervals (queen 1 from September 2007 through January 2008 and queens 2 and 3 from March through June 2009). Temperatures were recorded at the beginning of each 120-min observation session. Because of diminished honey stores, queen 1's colony was provided occasionally with 1M sucrose, *via* pipettes inserted through air vents at the top of the observation hive, during the months of December 2007 and January 2008. No sucrose was given during the 3-day observation sets. One observer (JJ) performed all of the observations for queen 1; queens 2 and 3 were shared equally by EH and JJ. To ensure subsequent uniformity of observations, the two observers worked together on 6 of the 12 observation sessions during the first (March) observation set for queen 2.

Because of the emphasis on tracking the behavior, we did not confirm that every occurrence of egg-laying behavior resulted in the production of an egg. However, occasional inspections never revealed failures by the queen to deposit an egg after performing the behavior. Therefore, the number of eggs/h laid by the queen was estimated for each monthly observation set by averaging the number of egg-laying behaviors recorded each hour during the 2-h observation sessions.

With rare exceptions the queen was constantly antennated, licked, and groomed by her attendants. For the purposes of this study these actions are not treated as queen behaviors because they apparently do not require active participation on the part of the queen. Also monitored were the numbers of worker bees attending the queen and any times that she was not visible (such as when the queen switched to the opposite side of the comb). The number of workers in the queen's retinue continually changed; therefore, only the first number noted during each successive 15 minutes of observation was used for subsequent analyses. The means were calculated from all of the observations at each particular time of day for all 3 days of the monthly observation set. Only workers that were facing the queen and antennating, licking, grooming, or feeding her were counted as members of her entourage.

2.5. Focal study 2: 24-h observations

Three different queens were observed during continuous 24-h sessions. All three colonies contained two full frames of bees (approximately 4000 workers). The first was queen 2 from focal study 1. Queen 2 was kept in the same colony as before and was approximately 1 year of age at the time of this study. The remaining two (designated queens 4 and 5) were unrelated to queens from any of the previous studies. Both were established in nucleus hives in April and transferred to observation hives during May; they were approximately 3 months of age at the beginning of this study. Queen 2 was monitored on three separate 24-h observation sessions spanning 28-29 June, 7-8 July, and 17-18 July, 2009. Queen 4 was observed during one 24-h session on 2-3 July, 2009 and queen 5 during three 24-h sessions on 5-6, 15-16, and 22-23 July, 2009. Queen 2 was located in the same site as in focal study 1 and queens 4 and 5 were located at sites C and B, respectively (previously used in scan-sampling study; Table 2); therefore, queen 2 was much more insulated from daily light and temperature fluctuations than

were queens 4 and 5. Temperatures were recorded hourly and weather conditions were noted continuously throughout the observation sessions. All of the observations were made by two observers (JJ and EH) in alternating 4-h shifts. The observations for queen 2 began at 20:00 h and ended at 19:59 h the next day for the first 24-h session and began at 06:00 h and finished at 05:59 h for the second and third sessions. For queens 4 and 5, all the sessions began at 18:00 h and finished at 17:59 h the following day. For uniformity and visual clarity, graphs depicting the 24 hours of each observation session were plotted from midnight to midnight.

2.6. Analyses

For the scan-sampling (multiple queen) study a chi-square goodness of fit test was used to determine if the number of occurrences of each behavior (pooled for all queens in each trial) were (1) distributed with equiprobability across four 6-hour periods, beginning with 07:00 h (corresponding approximately to sunrise) and (2) distributed with equiprobability between day and night (corrected for differences in duration). Differences between night and day in the relative frequencies of active and inactive behaviors were tested by a 2×2 chi-square contingency table analysis for each trial.

For focal study 1 the total number of minutes occupied by each behavior was compiled for each monthly set of observations for each queen. Each behavior was then assessed according to the proportion of time it occupied during the monthly observation sets. Potential relationships among the various behaviors (using data from all three queens) were tested for trends that were common to all three queens by using the Spearman rank-order correlation on these proportions. We used three different assessments of behavior with respect to time of day. First, each behavior (Table 1) was evaluated individually using data from all 3 days of each monthly observation set according to distribution among the four different observed times of day. For this purpose the

numbers of minutes occupied by the particular behavior for each observation time of day were submitted to a Kruskal-Wallis test across all four times of day ($df = 3$ in all cases). Second, each behavior was assessed for day vs. night differences in occurrence by comparing the minutes for all the daytime observation sessions with those for the nighttime sessions for all 3 days of the monthly observation set, using the Mann-Whitney test. If a significant difference was detected ($P < 0.05$), the behavior was then characterized as diurnal or nocturnal (i.e., highest level of performance of the behavior occurred during the day or night, respectively). Third, for an overall assessment of the presence or absence of diel rhythmicity, the numbers of minutes devoted to all the behaviors except standing were combined into a measure of 'activity' and the number of minutes occupied by standing was characterized as 'inactivity'. Potential differences between night and day were evaluated by comparing the minutes devoted to activity in all of the nighttime and daytime observation sessions using the Mann-Whitney test for each monthly observation set for each queen. Significant differences ($P < 0.05$) were interpreted as indicating overall diurnal or nocturnal rhythmicity (depending on which phase contained the higher proportion of active behaviors). Using the data from all three queens, Spearman rank-order correlation was used to test the relationships between the proportion of time engaged in active behaviors (1) during the day and the mean late-day ambient temperature, (2) during the night and the mean late-day ambient temperatures, and (3) during the night and the mean late-night ambient temperatures. Finally, the Kruskal-Wallis test was used to determine if the numbers of workers in the queen's retinue were distributed with equiprobability across all four observation times of day and the Mann-Whitney U-test was used to discern if retinue numbers were distributed with equiprobability between night and day.

For focal study 2 the number of minutes devoted to each behavior was compiled for each hour of each 24-h observation session. Then, each 24-h session was divided into four 6-h segments beginning with the hour closest to sunrise. A mean number of minutes devoted to each behavior was calculated from the hourly values within each of the four 6-h segments. For each behavior the Kruskal-Wallis test was used to check for differences among the four segments. The Mann-Whitney test was used to assess day vs. night differences in occurrence of each behavior: means for daytime (approximately 15 hours) and nighttime (approximately 9 hours) were calculated from the hourly values. To evaluate the presence or absence of overall diel rhythmicity the numbers of minutes devoted to walking, inspecting, and egg-laying were combined into an 'activity' measure and the number of minutes occupied by stationary behavior was characterized as 'inactivity.' Night and day activity levels were compared using the Mann-Whitney test for each 24-h observation session for each queen. Significant differences ($P < 0.05$) were interpreted as indicating overall diurnal or nocturnal rhythmicity (depending on which phase contained the higher proportion of active behaviors).

3. Results

3.1. Scan-sampling study

Scan-sampled observations of multiple queens indicated that all of the monitored behaviors (walking, stationary, inspecting, and egg-laying) occurred at all times of the day and night (Fig. 1). Using pooled data from all of the colonies, none of the behaviors showed variation in frequency of occurrence over four 6-h segments of the day-night cycle (χ^2 goodness of fit, $df = 3$, $P > 0.05$ in all cases) for any of the three trials. Similarly, none of the behaviors exhibited a significant difference in frequency between night and day (χ^2 goodness of fit, $df = 1$,

$P > 0.2$ in all cases). Furthermore, there were no significant differences in the frequency of active (combination of walking, inspecting, and egg-laying) and inactive (stationary) behaviors between night and day for any of the three trials (trial 1: $\chi^2 = 0.01$, $P = 0.920$; trial 2: $\chi^2 = 0.29$, $P = 0.590$; trial 3: $\chi^2 = 0.69$, $P = 0.406$; $df = 1$ in all cases). Although the queens were not analyzed as individuals in this particular study, it is apparent that stationary behavior and episodic interludes of active behaviors were distributed throughout both the day and night for every queen in all three trials (Fig. 1). This pattern was observed for a variety of colony sizes (Table 2) including exceptionally small ones (e.g., A1 in trial 1 and A2 in trial 3).

3.2. Focal study 1

In this study three queens were monitored individually. Each exhibited a rather limited behavioral repertoire consisting primarily of standing, walking, inspecting cells, and egg-laying, in order of prevalence. A relatively small proportion of the queens' behavior was occupied by feeding (between 1.0% and 11.8% of the time observed), begging for food (between 0% and 1.8%), and grooming self (between 1.6% and 3.4%). Common to all three queens was a high proportion of time engaged in standing. Furthermore, this proportion showed a substantial seasonal variation. For example, queen 1 was observed during the transition from late summer (September) activity to winter (January) inactivity: standing accounted for 44% of the observed behavior in September and 81% in January. In contrast, queens 2 and 3 were observed during the transition from late winter (March) to early summer (June): both showed a decrease in the proportion of time occupied by standing. For queen 2 standing declined from 72% of the observed behavior in March to 48% in June. Similarly, for queen 3 standing behavior occupied 42% of the observation time in March but only 21% in May and 30% in June.

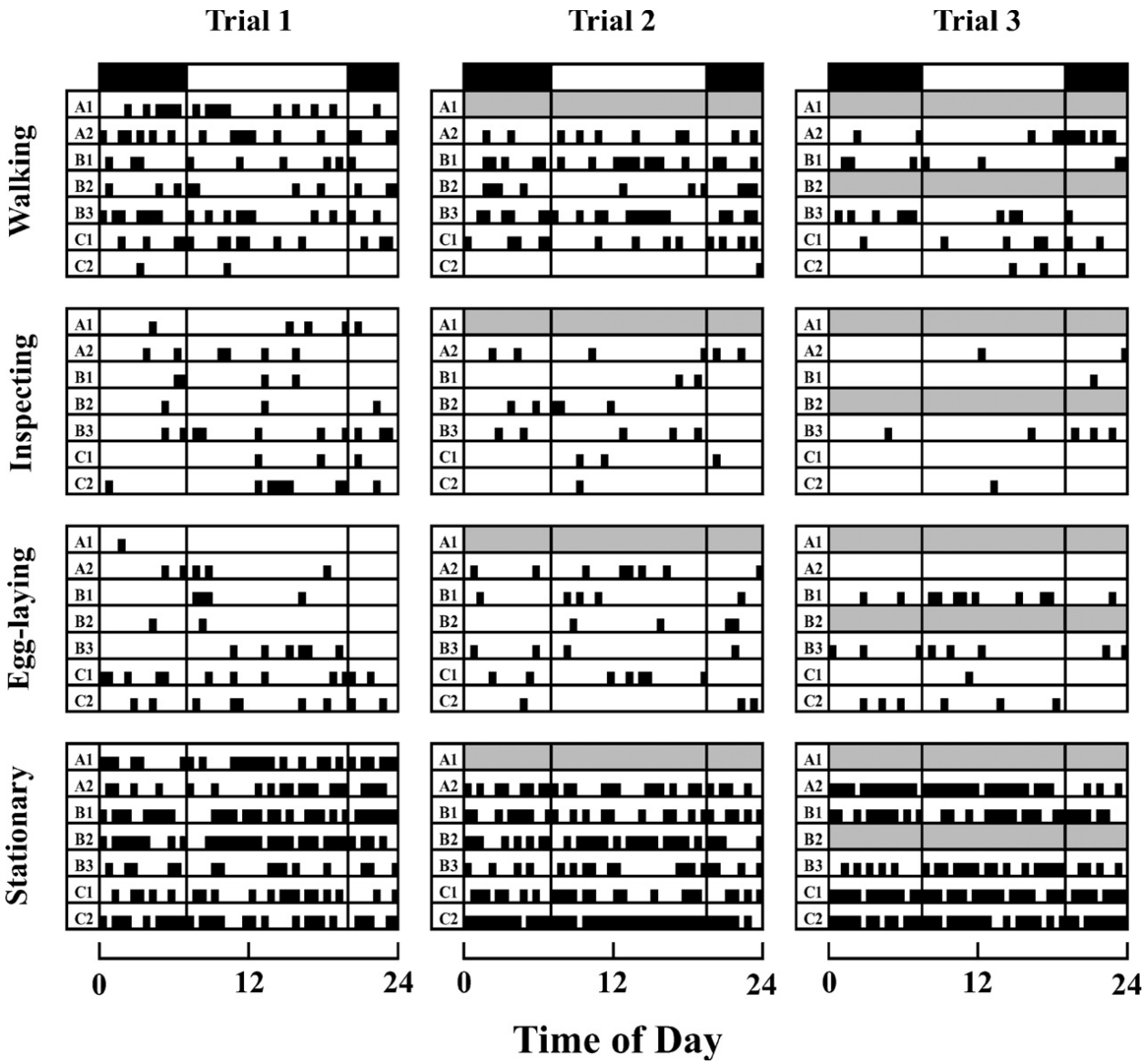

Figure 1 Absence of diel rhythmicity in queen behavior persists despite exposure to natural light-dark and temperature cycles. Graphs are composite representations of temporal behavioral performance in all three trials of the multiple queen study. Graphs depict scan sampled observations of first behavior observed; entire 24-h day was sampled (over a period of several days: 25 Aug.–2 Sept. for trial 1, 8-13 Sept. for trial 2, and 3-7 Oct. for trial 3) with a resolution of 30 min. Each horizontal line depicts performance of a particular behavior (within the panels for walking, inspecting, egg-laying, or standing) by one queen (labeled A1, A2, B1, etc.). Queens were obtained from three different sources and observed at three different sites (see text). Queen A1 was absent in trials 2 and 3; queen B2 was absent in trial 3. Natural day-night cycle for each trial is depicted above top panel. Vertical bars indicate presence of the behavior; blank spaces indicate its absence. All behaviors are performed at all times of the day and night.

With respect to the minute-to-minute temporal patterning of behaviors (Fig. 2), the queens typically showed short bouts of activity (primarily walking, inspecting, and egg-laying) that were separated by periods of standing behavior. Begging for food, feeding, and grooming self behaviors often took place within the standing bouts. Similar patterns of standing alternating with bouts of activity were observed by Seeley (1979).

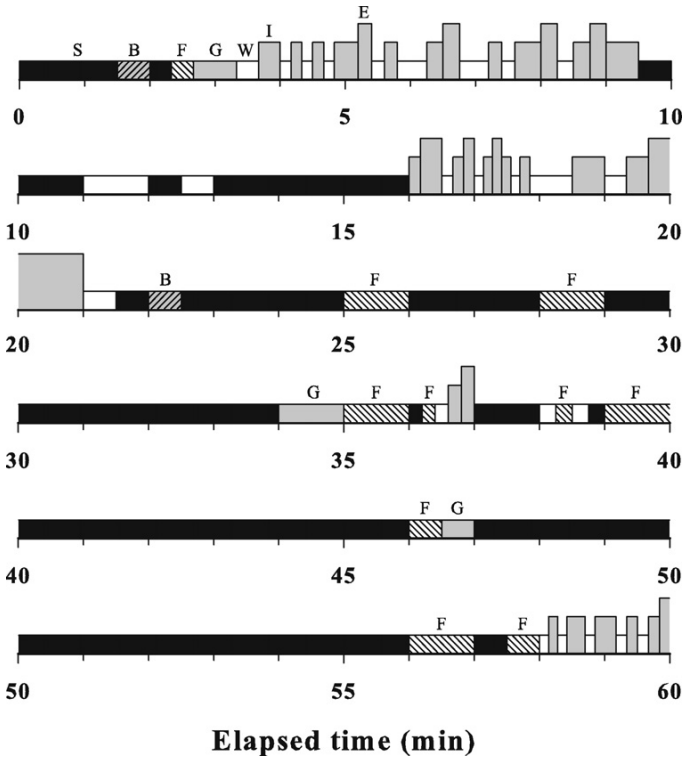

Figure 2 One hour in the life of a queen: time-line showing minute-by-minute patterning of behavior. Observations were taken from 04:45-05:45 on 18 March, 2009 for queen 3. Numbers indicate time, in minutes. Behaviors are as follows: S-standing, B-begging for food, F-feeding, G-grooming self, W-walking, I-inspecting cells, E-egg-laying. Only the first occurrence of S (black, lowest height), W (white, lowest height), I (gray, middle height), and E (gray, tallest height) are labeled with letters; all other behavioral occurrences are labeled throughout the time-line. Note that short bouts of activity alternate with somewhat longer bouts of inactivity (standing) and also that the occurrences of begging for food, feeding, and grooming self are embedded within the standing bouts.

There were a limited number of consistent seasonal trends shared by all three queens with respect to relationships between behaviors. The proportion of time engaged in standing behavior was negatively correlated with the proportions for walking, inspecting, and egg-laying (Spearman rank-order correlation: r_s = -0.707, -0.962, and -0.901, respectively; n = 13, P<0.01 in all cases). Thus, as the proportion of time occupied by standing behavior increased with the approach of winter or decreased with the approach of summer, three of the remaining six behaviors exhibited a significant change in frequency of occurrence in the opposite direction. Using queen 1 as an example, over three consecutive monthly observations (September, October, and November), standing behavior occupied about 44%, 63%, and 80% of the observed time

while egg-laying behavior accounted for 13.4%, 5.5%, and 0%, respectively. As might be expected, inspecting and egg-laying behaviors showed a significant positive correlation (Spearman rank-order correlation: $n = 13$, $r_s = 0.962$, $P<0.0001$). Among all the remaining pairwise comparisons of behaviors, none showed a significant correlation.

A remarkable feature exhibited by all three individually monitored queens was the continuous occurrence of each behavior at all four observed times of day (early and late day, early and late night). This around-the-clock performance of all behaviors persisted despite seasonal changes in the frequencies of occurrence of the behaviors relative to each other (Fig. 3). A significant difference in the minutes apportioned to a particular behavior across the four sampled times of day occurred in only 4 out of 89 possible instances (Table 3; two cases showing total absence of a behavior were excluded). A significant difference between night and day in the amount of time allotted to the performance of a particular behavior occurred in only 7 out of 89 possible cases (Table 3): there were no apparent consistencies in these relatively rare occurrences with respect to queen, season, or behavior.

Table 3 Consistency of behavioral performance (tabulated in minutes) with respect to time of day for all three individually monitored queens in focal study 1 over all monthly observation sessions. Top of each panel: individual behaviors were examined for differences in performance across all four observation times of day by Kruskal–Wallis test; P values are reported here. Significant deviations from equiprobability ($P<0.05$) are indicated in bold. Bottom of each panel: individual behaviors examined for day vs. night differences by Mann–Whitney test [NS: no significant difference ($P>0.05$) between night and day, D: $P<0.05$ with highest activity during the day, N: $P<0.05$ with highest activity at night] (– no occurrence of the behavior during the observation session).

Behavior	Queen 1					Queen 2				Queen 3			
	Sept	Oct	Nov	Dec	Jan	Mar	Apr	May	June	Mar	Apr	May	June
Standing	0.933	0.826	**0.033**	0.988	0.644	0.789	0.668	0.347	0.090	0.066	0.092	0.433	0.147
	NS	NS	NS	NS	NS	NS	NS	NS	NS	**N**	NS	NS	NS
Walking	0.218	0.432	0.161	0.578	0.392	0.863	**0.013**	0.789	0.442	0.319	0.376	0.758	0.863
	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Inspecting	0.953	0.200	0.408	0.951	0.392	0.589	0.147	0.863	**0.022**	0.057	0.121	0.983	0.129
	NS	NS	NS	NS	NS	NS	NS	NS	**D**	**D**	NS	NS	NS
Egg-laying	0.319	0.340	–	0.313	0.679	0.985	0.392	0.392	0.108	0.223	0.118	0.203	0.063
	NS	NS	–	NS	NS	NS	NS	NS	**D**	NS	NS	NS	NS
Feeding	0.400	0.157	0.218	0.414	0.282	0.668	0.814	0.192	0.057	0.516	0.516	0.147	0.157
	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Begging for food	0.913	–	0.382	0.875	0.863	0.863	**0.044**	0.123	0.319	0.304	0.972	0.144	0.150
	NS	–	NS	NS	NS	NS	**N**	NS	NS	NS	NS	NS	**N**
Grooming self	0.147	0.835	**0.034**	0.223	0.218	0.259	0.092	0.058	0.622	0.183	0.706	0.218	0.183
	NS	NS	NS	NS	NS	NS	NS	**D**	NS	NS	NS	NS	NS

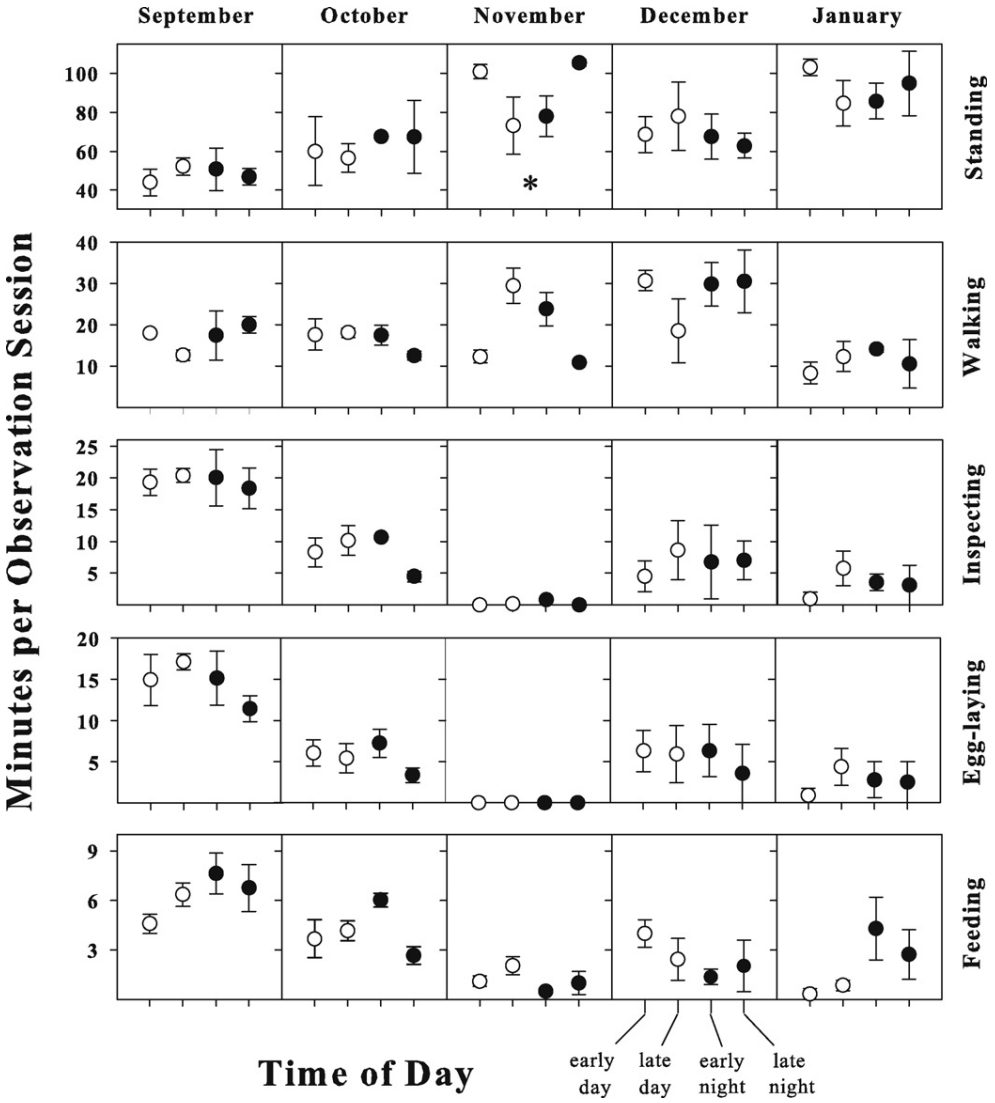

Figure 3 Performance of individual behaviors by honey bee queens is distributed across all four observed times of day, during different times of the year. The mean number of minutes devoted to individual, overt behaviors is indicated, left to right, for early and late day (white bars) and for early and late night (dark bars) observation sessions (monitored over three consecutive days, once per month) for queen 1 (queens 2 and 3 are not shown but demonstrate similar levels of variability). Five behaviors are depicted (rows: standing, walking, inspecting, egg-laying, and feeding) over five consecutive months (columns). * denotes significant ($P < 0.05$) deviation from equiprobability of performance of a particular behavior among the four observed times of day. There were no diurnal or nocturnal patterns associated with any observed behavior.

To determine if honey bee queens show diel (day vs. night) rhythmicity in activity, standing behavior was used as an indicator for inactivity and the combination of all other behaviors was treated as the assay for activity (Moore et al., 1998). As demonstrated with the individual behaviors, the predominant pattern was no significant difference between night and

day with respect to minutes devoted to activity: the only significant occurrence was in March for queen 3 (diurnal: Mann-Whitney test, U= 4.0, $P = 0.025$).

Egg-laying rates, simply estimated from the occurrences of egg-laying behavior, varied by queen and by season. The rates for queen 1 for the months of September through January were 28, 12, 0, 1, and 7 eggs/h, respectively. For the months of March through June the rates were 2, 13, 13, and 21 eggs/h for queen 2 and 16, 32, 31, and 21 eggs/h for queen 3. These rates compare favorably to the 34-36 eggs/h observed by Schmickl et al. (2003) during the summer for a 2-year-old queen housed in an observation colony containing 13,000 workers.

There was an inconsistent relationship between day-night differences in ambient temperature and the presence of day-night differences in queen activity levels (Fig. 4). The absence of a significant difference in activity between day and night occurred in all three queens despite large differences in daytime and nighttime ambient temperatures (e.g., all monthly observations for queen 1; March observations for queen 2; and April through June observations for queen 3). On the other hand, an apparent relationship did exist between ambient temperature and overall activity levels. For example, the proportion of time occupied by active behaviors during the day was significantly correlated with daytime temperature (Spearman rank-order correlation, $n = 13$, $r_s = 0.923$, $P < 0.0001$). Interestingly, the proportion of time occupied by active behaviors during the night was also significantly correlated with daytime temperature ($r_s = 0.857$, $P < 0.001$) but not with nighttime temperature ($r_s = -0.253$, $P = 0.404$).

In parallel with the lack of consistent diel rhythmicity in behavioral performance, there were no consistent differences in the number of workers in the queen's retinue with respect to time of day (Fig. 5). In the case of queen 1, for example, there were no significant differences

among the four sampled times of day except for January (Kruskal-Wallis; H = 16.01, *df* = 3, *P* = 0.001). Significant differences in retinue size with time of day occurred only during the March observations for queen 2 (Kruskal-Wallis; H = 16.7, *df* = 3, *P* < 0.001) and the June observations for queen 3 (Kruskal-Wallis; H = 9.51, *df* = 3, *P* = 0.023). Of these three cases diurnal rhythmicity in retinue number was detected in the January observations for queen 1 (Mann-Whitney test, U = 579.5, *P* = 0.0001) and the March observations for queen 2 (Mann-Whitney test, U = 583.5, P < 0.0001) but not for the June observations for queen 3 (Mann-Whitney test, U = 1056, *P* = 0.717). Neither of the two cases of diurnal rhythmicity in retinue size coincided with diurnal rhythmicity in queen activity.

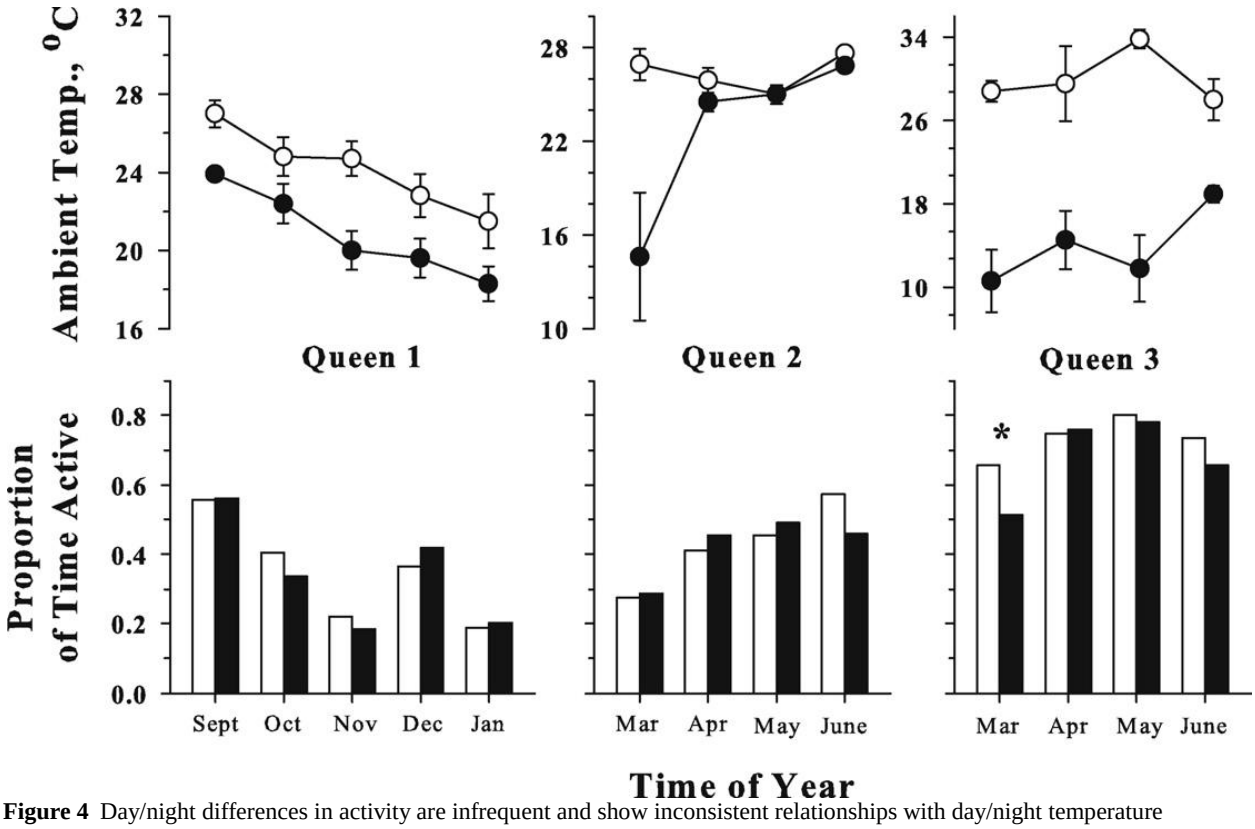

Figure 4 Day/night differences in activity are infrequent and show inconsistent relationships with day/night temperature oscillations. Bottom: proportion of time devoted to active behaviors during the daytime (white bars) and nighttime (black bars) observations for each monthly observation session for each queen. *Active behaviors* is defined as the performance of all behaviors except standing; * denotes significant difference (*P* < 0.05) between day and night in the amount of time devoted to active behaviors. *Top*: mean late day (white circles) and late night (black circles) ambient temperatures (± SEM) present during the corresponding monthly behavioral observations.

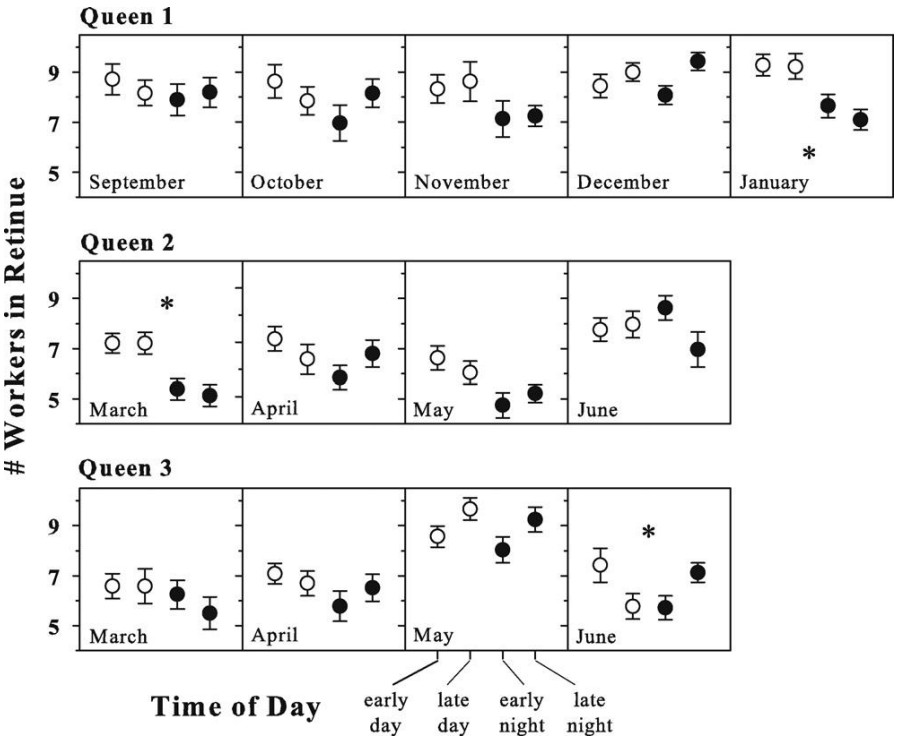

Figure 5 The size of the queen's retinue shows little variation with time of day. Mean number of workers (± SEM) are shown, from left to right, for early day and late day (white circles) and early night and late night (black circles) observation times of day.

3.3. Focal study 2

Three queens were monitored continuously during 24-h observation sessions. The first of these, queen 2 from focal study 1, was observed on three separate occasions over a span of 20 days: the queen's behaviors showed remarkably little variation with respect to time of day (Fig. 6). During the first two 24-h observation sessions, none of the observed behaviors (stationary, walking, inspecting, and egg-laying) showed significant deviation from equiprobability of occurrence among four 6-h segments of the day beginning with sunrise (Kruskal-Wallis, $df = 3$, $P > 0.05$ in all cases). During the third observation session only egg-laying behavior showed significant variation with respect to time of day (Kruskal-Wallis, $H = 9.165$, $df = 3$, $P = 0.027$). No behavior exhibited a significant difference between day and night performance during any of the three observation sessions (Mann-Whitney, $P > 0.10$ in all cases), nor were there any significant differences between day and night in overall activity levels (Mann-Whitney, $P > 0.2$ in all cases). The mean temperatures and the minimum-to-maximum temperature ranges (in ^{o}C) were 28.7 (27.8 to 29.4), 27.4 (25.6 to 28.9), and 26.6 (25.0 to 27.8) for the first, second, and

third sessions, respectively. Local weather conditions were similar (mostly cloudy) for all three sessions.

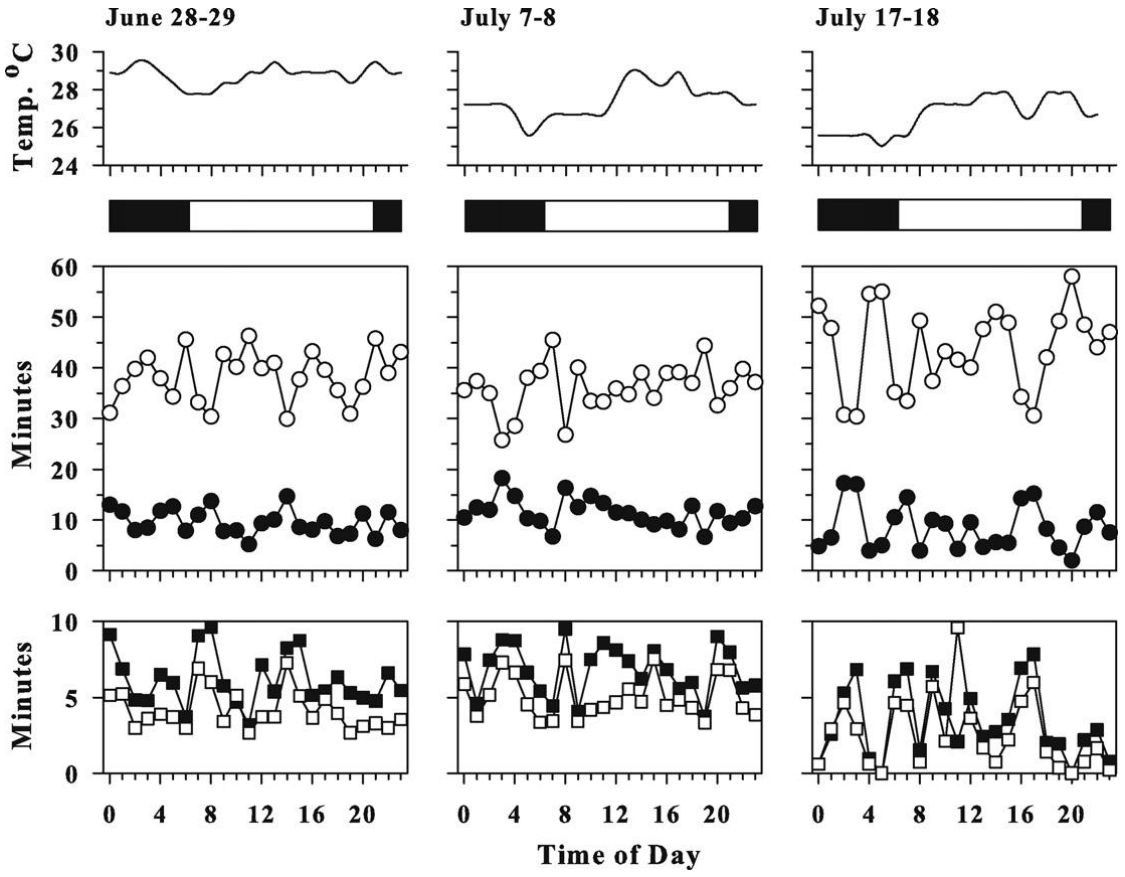

Figure 6 Minutes devoted to different behaviors by queen 2, compiled hourly during three different 24-h observation sessions. Each column portrays one session (dates are indicated above each column). Top panel: ambient temperatures recorded adjacent to the observation hive throughout the observation session. Natural photoperiod is indicated by bars immediately below temperature profiles. Middle panel: minutes occupied by stationary (white circles) and walking (black circles) behaviors. Bottom panel: minutes occupied by inspecting (black squares) and egg-laying (white squares) behaviors. Time of day for each column is indicated below the bottom panel. No diurnal or nocturnal patterns were detected.

A second queen (queen 4) was monitored during one 24-h observation session (not shown). Only walking behavior showed significant variation with respect to time of day (Kruskal-Wallis, $H = 9.167$, $df = 3$, $P = 0.027$) and only egg-laying behavior exhibited a significant difference between day and night (Mann-Whitney, $U = 25.0$, $P = 0.011$). However, there was no significant difference in overall activity level between the daytime and nighttime

hours (Mann-Whitney, $U = 64.0$, $P = 0.835$). The mean temperature was 19.7 ^{o}C and the minimum-to-maximum range was 15.0 to 25.0 ^{o}C over the observation session. Weather conditions varied from scattered clouds to mostly cloudy.

A third queen (queen 5) displayed evidence for diel rhythmicity during two out of three 24-h observation sessions (Fig. 7). During the first session, none of the behaviors showed significant variation among the four 6-h segments across the day (Kruskal-Wallis, $df = 3$, $P > 0.10$ in all cases) nor between day and night (Mann-Whitney, $P > 0.10$ in all cases). Furthermore, there were no significant differences in overall activity levels between day and night (Mann-Whitney, $U = 48.0$, $P = 0.245$). In contrast, during the second 24-h observation session (10 days later), both stationary and walking behaviors (Kruskal-Wallis, $df = 3$: $H = 14.5$, $P = 0.002$ and $H = 11.7$, $P = 0.008$, respectively) showed significant variation among the four different times of day. Stationary, walking, and inspecting behaviors exhibited significant variation between day and night (Mann-Whitney: $U = 24$, $P = 0.009$; $U = 18$, $P = 0.003$; and $U = 19$, $P = 0.004$) as did the overall activity levels (Mann-Whitney: $U = 24$, $P = 0.009$; diurnal). Similar to the second session, during the third 24-h observation session (7 days after the second session), stationary, walking, and inspecting behaviors varied significantly across the four different times of day (Kruskal-Wallis, $df = 3$: $H = 13.02$, $P = 0.005$; $H = 8.99$, $P = 0.029$; $H = 9.42$, $P = 0.024$). Stationary and walking behaviors varied significantly between day and night (Mann-Whitney: $U = 3.0$, $P < 0.001$; $U = 8.0$, $P < 0.001$) as did the overall activity levels (Mann-Whitney: $U = 9.5$, $P < 0.001$; diurnal). Interestingly, the temperature profiles showed minimal differences among the three observation sessions. The mean temperatures and the minimum-to-maximum temperature ranges (in ^{o}C) were 22.4 (20.5 to 26.1), 24.3 (22.2 to 27.8), and 23.1(21.1 to 27.2) for the first, second, and third sessions, respectively. Because the sessions

all occurred within a span of 17 days, the natural photoperiods were virtually identical. Local weather conditions were remarkably similar across all three sessions, showing little variation from overcast to mostly cloudy.

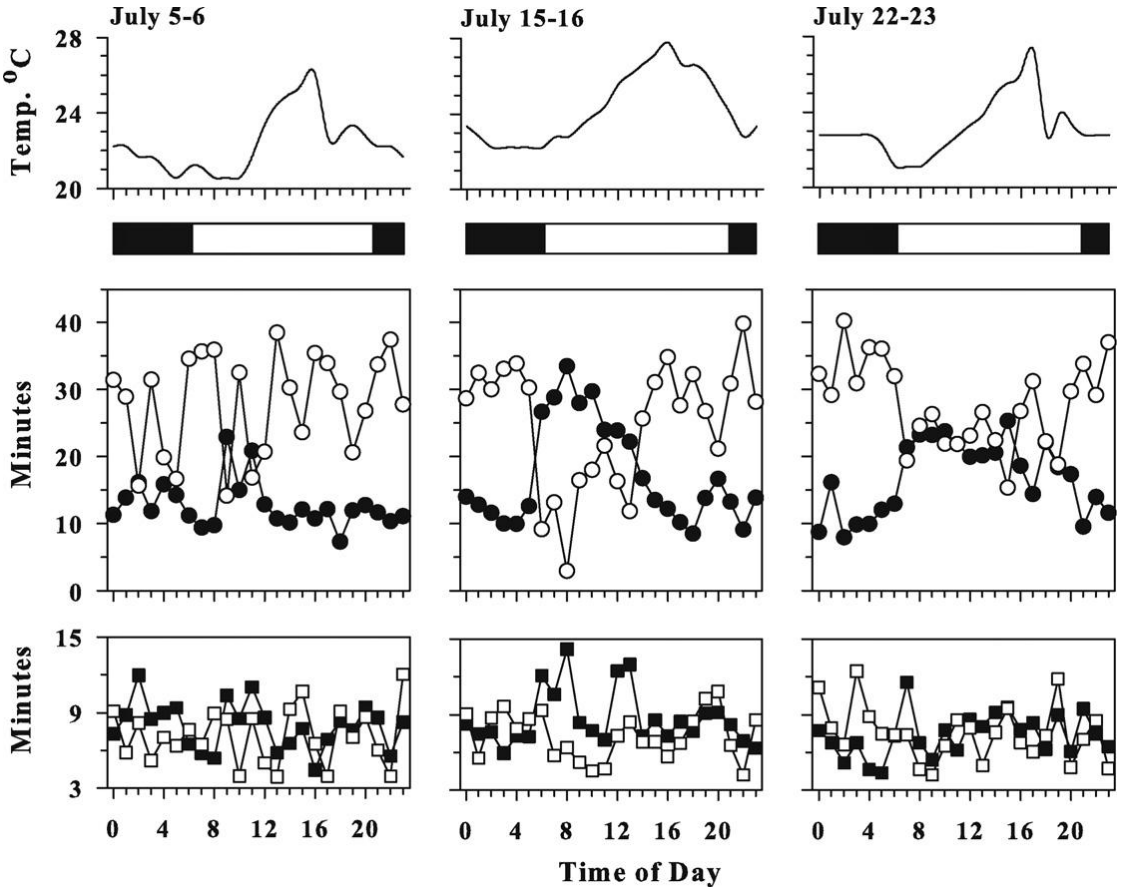

Figure 7 Minutes devoted to different behaviors by queen 5 compiled hourly during three different 24-h observation sessions. Each column portrays one session (dates are indicated above each column). Top panel: ambient temperatures recorded adjacent to the observation hive throughout the observation session. Natural photoperiod is indicated by bars immediately below temperature profiles. Middle panel: minutes occupied by stationary (white circles) and walking (black circles) behaviors. Bottom panel: minutes occupied by inspecting (black squares) and egg-laying (white squares) behaviors. Time of day for each column is indicated below the bottom panel. Diurnal activity patterns were detected during the second and third sessions, but not the first.

4. Discussion

Most of the evidence gathered in this report suggests that within the social confines of the colony the behavior of mated honey bee queens is arrhythmic with respect to time of day. The evidence for arrhythmicity is as follows: (a) Every behavior in the queen's repertoire,

notably including egg-laying, was observed at all hours of the day and night (Figs. 1, 3, 6, and 7). (b) In focal study 1 the vast majority of cases (85 of 89 tests) revealed no significant differences in minutes committed to any type of behavior among the four observed times of day (Table 3). Also, none of the behaviors examined in the scan-sampling study (Fig. 1) showed a significant deviation from equiprobability of occurrence across different times of day. (c) Testing for the presence of day-night differences in the performance of individual behaviors revealed relatively few such instances (7 of 89 tests) in focal study 1 (Table 3) and none in the scan-sampling study. It is important to note that in focal study 1 there were no cases in which an individual behavior performed by a queen maintained a diel periodicity for more than one monthly observation session. Egg-laying, for example, exhibited a significant day-night difference in only one observation session (June) for queen 2 only. Walking and feeding behaviors failed to show any significant day-night difference in any observation session (Table 3). (d) To provide a more encompassing measure of behavioral rhythmicity, the amount of time devoted to standing behavior was taken as an indication of inactivity (i.e., rest) and the amount of time committed to all other behaviors was considered to be an indication of activity. In focal study 1 only one out of 13 monthly observation sets showed a significant difference in activity between night and day -- the March observation session for queen 3 (Fig. 4). In the scan-sampling study none of the trials demonstrated a significant day-night difference in the relative amounts of activity and rest. (e) The predominant absence of day-night differences in activity levels often occurred despite relatively large differences in temperature between night and day (Fig. 4). Interestingly, the proportion of time engaged in active behaviors during both day and night was correlated with the mean daytime but not nighttime ambient temperatures. (f) The scan-sampling study revealed an apparent absence of diel rhythmicity in all of the behavioral measures in all three trials despite

the fact that all of the observation colonies were exposed to natural, ambient changes in both temperature and light levels and thus were confronted with two potential daily entraining cycles (Aschoff, 1981). (g) In the vast majority of cases the number of workers in the queen's retinue failed to deviate significantly from equiprobability with respect to time of day (Fig. 5), suggesting that the attractiveness of the queen to workers is also arrhythmic. (h) Two out of three queens monitored in 24-h sessions (focal study 2) exhibited arrhythmic activity patterns.

In contrast to the evidence for behavioral arrhythmicity outlined above, one queen in focal study 2 displayed obvious diel rhythmicity during two out of three 24-h observation sessions (Fig. 7). The underlying reason for arrhythmicity in the first session but diurnal rhythmicity in the other two sessions is not apparent: all were conducted at the same location within a span of 17 days. As shown in Fig. 7, the temperature changes encountered by the colony were similar in temporal profile for all three sessions. The amplitudes were slightly lower in the first session relative to the second (by about 2^{o} C) and the third (by about 1^{o} C). Because the weather conditions were similar for all three sessions (overcast or mostly cloudy), the light levels also were comparable. In both the second and third observation sessions walking behavior showed an increase (and stationary behavior a decrease) in minutes/h that anticipated both the sunrise as well as the morning elevation in temperature. Inspecting behavior was significantly diurnal during the second observation session but not the first or third. Only egg-laying behavior was arrhythmic for all three 24-h observation sessions.

The diurnal activity pattern during the second and third observation sessions for queen 5 stand in contrast to the arrhythmic patterns produced by queens 2 (Fig. 6) and 4 (not shown). The factors responsible for the differences are not clear. Although queen 5 experienced larger amplitude (about 6^{o} C) day-night temperature fluctuations than those encountered by queen 2

(about 3^{o} C), the ambient temperature variation experienced by queen 4 was even greater (10^{o} C). While the arrhythmic queen 2's colony was exposed to natural light only through the hive entrance, the colonies for the arrhythmic queen 4 and the sometimes rhythmic queen 5 both received some daytime indirect light in addition to illumination through the entrance. Furthermore, there were no easily discriminable differences among the weather conditions present during any of the 24-h observation sessions: all were characterized by cloudiness throughout the day and night.

In most studies of circadian rhythms in animals the isolated individual partitions its time into active and inactive (resting) phases. For diurnal organisms, including honey bee foragers (Moore and Rankin, 1985; Frisch and Aschoff, 1987), this usually entails a more-or-less continuous active phase positioned primarily during photophase of LD cycles (or subjective day under constant conditions) as well as a more-or-less continuous inactive phase positioned primarily during the scotophase (or subjective night under constant conditions). Our observations indicate that within the colony the mated honey bee queen does not segregate its behavior into separate, daily active and resting phases. Instead, its rest is scheduled throughout all hours of the day and night; in fact, activity and rest are thoroughly intertwined (Fig. 2). This arrhythmic temporal pattern is similar to that exhibited by young worker honey bees in the colony (Crailsheim et al., 1996; Moore et al., 1998; Shemesh et al., 2007): before they become foragers, workers perform their age-specific tasks including brood care around-the-clock. However, as the worker approaches the onset of foraging, at about 3 weeks of age, it increases its frequency of resting behaviors during the night while continuing to perform its tasks at all times of the day and night (Moore et al., 1998). Once foraging status is reached, the worker is unequivocally diurnal, scouting or collecting resources during the daylight hours and resting at

night (Kaiser and Steiner-Kaiser, 1983; Kaiser, 1988; Sauer et al., 2003; Klein et al., 2008). Our observations, with one exception, suggest that the behavior of the mated honey bee queen may be driven by a temporal program similar to that of young workers – rest and activity are both arrhythmic. Although the relative proportions of activity and rest change with the seasons (Figs. 3 and 4), the fundamental temporal pattern of behavioral arrhythmicity persists.

Free et al. (1992) reported an absence of diel periodicity for three different factors relevant to queen honey bee behavior – the number of workers in the queen's retinue, the distance traveled per unit time, and the numbers of eggs laid. Our results are similar. We report that there is no consistent diel periodicity in (1) the number of workers attending to the queen, (2) the amount of time committed by the queen to walking behavior, and (3) the amount of time devoted to egg-laying behavior. By examining the potential for periodicity in all of the behaviors in the queen's repertoire, we have extended the findings of Free et al. (1992) and eliminated the possibility that some individual behaviors are consistently rhythmic while others are not. Furthermore, by using standing behavior as an indicator for rest and all other behaviors as indicators for activity, we have a metric for detection of putative sleep-wake cycles (Moore et al., 1998). Again, with one exception, our results indicate an absence of diel periodicity.

Our results provide an interesting contrast to the findings from two other studies of circadian rhythms in queen honey bees. Moritz and Sakofski (1991) examined the free-running circadian rhythm of oxygen consumption in groups of 150 worker honey bees. In response to introduction of individual queens that were kept in colonies 8 hours out of phase with the test groups, the worker rhythms showed an average phase shift of 1.4 hours in the direction of the queens' activity rhythm on the first day after the transfer. There were no phase-shifts on subsequent days. Introduction of individual worker bees failed to generate significant phase-

shifts. While it appears certain that the introduced queen is capable of eliciting a transient phase-shift, it is difficult to discern its root cause. Attributing the phase shift to the influence of the queen's activity rhythm is problematic: first, the queen's rhythm was not directly measured but rather inferred from the peak activity (i.e., foraging) of the host colony and second, as shown in the present study, the queen may or may not exhibit overt rhythmicity. On the other hand, locomotor activity in individually isolated honey bee queens (both virgin and mated) has been shown to entrain to LD cycles and to free-run under constant conditions (Harano et al., 2007). One parsimonious explanation that encompasses all of these findings, including those of the current study, is that unmated and mated queens possess the capacity for expressing behavioral rhythmicity, but that capability is masked or inhibited in mated queens within the social setting of the colony. Presumably, once the queen is removed from colonial influences (as yet undetermined), the rhythmicity may once again be expressed. Perhaps, in the case of queen 5 (which showed rhythmicity on two occasions and arrhythmicity on another), the unknown factors inhibiting rhythmicity or the queen's sensitivity to those factors were relatively weak. According to this scenario honey bee queens may show the same rhythmic plasticity demonstrated by nurse bees; these workers are arrhythmic within the colony but demonstrate free-running circadian rhythms of locomotor activity when removed from the colony and isolated under constant conditions (Shemesh et al., 2007). A direct test of this hypothesis would seem to be a logical next-step in understanding the queen circadian system.

It has yet to be determined if virgin honey bee queens show diel rhythmicity within the colony. Certainly, virgin queens must consult a working circadian clock in order to navigate to and from drone congregation areas. However, does this clock mechanism also contribute to the patterning of behavior inside the hive at this stage in the queen's development? The prototype of

displaying rhythmicity early but becoming arrhythmic after mating has been observed in the queens of several ant species (McCluskey, 1992; Sharma et al., 2004a). The predominant absence of diel rhythmicity in egg-laying behavior by honey bee queens observed in this study fits well with the previous finding of around-the-clock performance of brood care by worker bees (Moore et al., 1998). The findings of the present study will help set the stage for future investigations into the ontogeny and plasticity of circadian rhythms in honey bee queens as well as the control mechanisms underlying the dynamic interplay between queen and worker behaviors.

Acknowledgements

We thank K. Joplin, C. Cannon, J. Kintner, M. Levine, T. Jones, and A. Mains for providing study sites for the observation hives and B. Van Nest, S. Boyd, and A. Edge for their substantial help in all aspects of this project. The work was supported by the William and Nina Fraley Award (Dept. of Biological Sciences, East Tennessee State University) to JNJ and U.S. Department of Agriculture grant 2006-35302-17278 to DM.

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CHAPTER 3

DISCUSSION

By examining her activities using three types of experimental conditions, we can conclude that the mated queens in these studies show consistently inconsistent behavior patterns, supporting our initial hypothesis that the queen's actions are random. None of the 13 queens used in our experiments showed consistent diel behavior patterns. Although one queen in the 24-h study showed 2 instances of diel behaviors (inspecting cells and being stationary), this pattern was only repeated in one or 2 of the observation days, respectively. In the 2-h observations, the queens did not show a consistent diel pattern with respect to month, queen, or behavior.

Under our experimental conditions, it was difficult to determine the substrate that the queen was on at all times, so we were unable to determine if substrate plays a role in activity. Based on her activity patterns, she spends most of her time standing with smaller bouts of inspecting, laying, grooming, and feeding (Figure 2). We have observed the queen performing most behaviors on all types of substrate, and it appears that she does not continuously lay eggs as long as cells are available, but instead often stops or walks away when clear cells remain. It is unclear how she is able to determine if a cell is suitable for egg-laying, as she sometimes inspects a cell, passes by it, and then moments later inspects it again, this time, finding it suitable enough for egg-deposition. The focus of our experiments was the queen and her behavior, so it would have been impossible to watch every cell she inspected to determine if workers cleaned them between her inspections. Throughout all the hours of observation, we never observed the queen laying eggs in cells containing pollen, nectar, or honey, though occassionally she laid eggs repeatedly in the same cells.

These experiments did not fully address the internal programming hypothesis. Instead, we lumped her activities together and looked at the total time spent doing each thing. More experiments would be needed to confirm or deny this.

We did not find results for a consistent diel or nocturnal pattern in her behavior (Figures 1, 3, 4, 6, 7, Table 3). Of the queens that did show either a diurnal or nocturnal rhythm, they were not consistent. In focal study 1 the queen's behavior differed from equiprobability across all 4 observation time periods for only 1 behavior and 1 month but showed no diurnal or nocturnal behavior pattern (Figure 3). Queens 2 and 3 showed inconsistent differences with respect to behavior and month (Figure 4). In focal study 2 queen 2 had no diurnal or nocturnal differences (Figure 6), while queen 5 had 2 instances of diurnal walking behavior and 1 instance of diurnal inspecting behavior. Even when there were differences, they were not consistent among months, queens, or behaviors.

Although the scan-sampling study only shows a resolution of 30 minutes, we do not see any patterns in the behaviors noted for each queen. The more in-depth 24 hour focal study does not show any consistent patterns at all. Only 1 of 3 queens showed any behavioral pattern, but it was inconsistent among the 3 observation time periods.

Based on our experiments, the most plausible explanation is that the queen is arrhythmic with respect to night and day. The queens perform each behavior both day and night (Figure 1) and, although they show longer bouts of standing, still intermingle standing with each of the other behaviors (Figure 2).

Although we were not able to perform 24-hour continuous observations all year round, we were able to have 2-hour continuous observations for all months except three (February, July, and August), so this gives us a general idea of what the queen does all year long. We do see that

the colony slows down egg production during the winter and increases it during spring (Figures 1, 3, and Figure 8, Appendix 1) as we would expect. During winter months, the colony is less active, not flying out of the hive to forage and huddling together to stay warm. Interestingly, we see that the queen continues to inspect cells and lay eggs throughout the winter months. For an unknown reason, the queen in our study did not lay eggs during the month of November, but because she resumed egg-laying in December and January, both of which had lower daytime and nighttime ambient temperatures (Figure 4), we have no reason to believe this lack of egg-laying is temperature or season-dependent.

APPENDIX

Percentage of Time Spent in Each Behavior for Focal Study 1

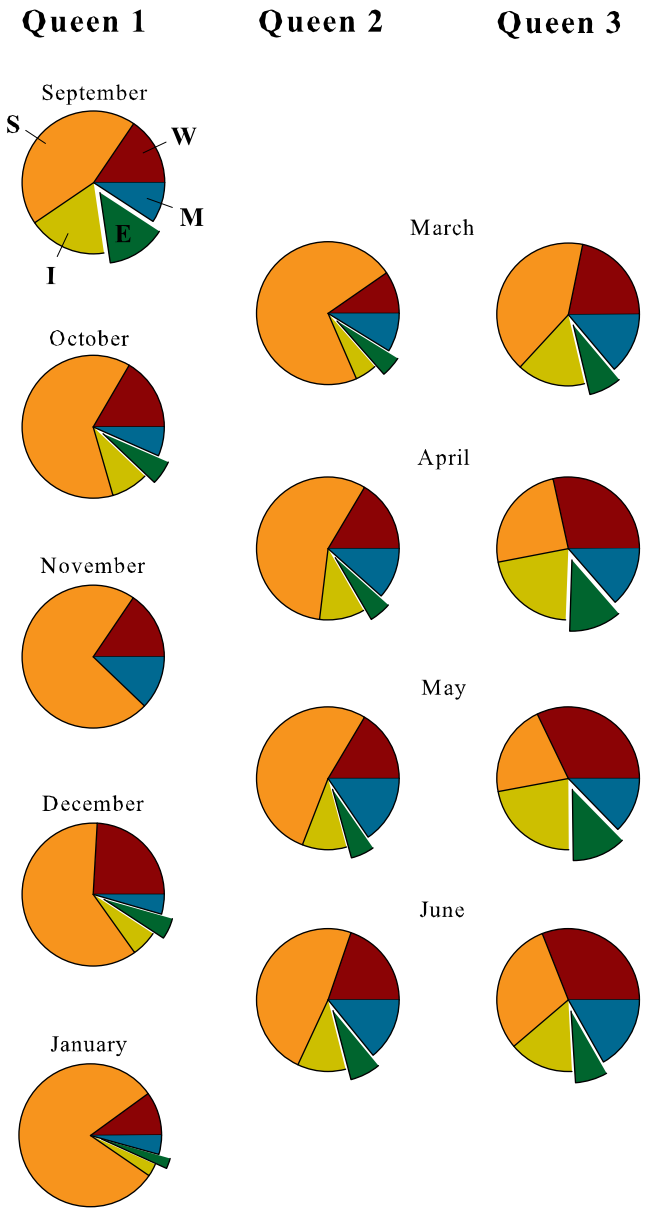

Figure 8 Percentage of time spent in each behavior for focal study 1. S: standing, W: walking, M: maintenance (combined behaviors of grooming self, feeding, and begging), E: egg-laying, I: inspecting cells. The majority of each queen's time is spent standing, although all behaviors occur in each month except November. The queen spends more time standing with the approaching winter, and more time inspecting and egg-laying as spring approaches.

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Honors and Awards:

Judge, University School Science Fair, University School, Johnson City, TN, 2008.

William Harvey Fraley and Nina M. Fraley Award for Graduate Research in Organismal and Cellular Biology, Department of Biological Sciences, East Tennessee State University, 2008.

Affiliations:	American Animal Hospital Association
	American Association for the Advancement of Science
	Entomological Society of America
	Society for Neuroscience
	Society of Veterinary Behavior Technicians