

The Influences of Illumination Regime on Egg-laying Rhythms of Honey Bee Queens

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Abstract Honey bee queens show extreme fecundity, commonly laying more than a thousand eggs in a single day. It has proven challenging to study the temporal organization of egg-laying behavior because queens are typically active around the clock in the dark cavity of a densely populated nest. To contend with this challenge, we developed two novel methods allowing detailed monitoring of queen activity and egg laying. We first adapted a high-resolution, continuous, tracking system allowing to track the position of barcode-tagged queens in observation hives with colonies foraging outside. We found that the queen is active ~96% of the day with typically no diurnal rhythm. Next, we developed a new laboratory procedure to monitor egg laying at single egg resolution under different light regimes. We found that under constant darkness (DD) and temperature conditions, queens laid eggs with no circadian rhythms. Queen fecundity was severely reduced under constant light (LL). Under a 12:12 illumination regime, queen fecundity was comparable to under constant darkness, with a higher number of eggs during the light phase. These daily rhythms in egg laying continued when these queens were released to DD conditions, suggesting that egg-laying rhythms are influenced by endogenous circadian clocks. These results suggest that honey bee queens are active and lay eggs around the clock with no diurnal rhythms. Light has complex influences on these behaviors, but more studies are needed to determine whether these effects reflect the influence of light directly on the queen or indirectly by affecting workers that interact with the queen.

Keywords honey bee, queen, egg laying, circadian rhythm, illumination regime, fecundity

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Social insects are characterized by a division of labor in reproduction where only a single or a few individuals (typically queens) lay eggs, whereas all other individuals (workers) perform all other non-reproductive tasks in the colony (Wilson, 1971). In eusocial insects from the order Hymenoptera (bees, wasps, and ants), the queen is the only mated female that can lay fertile diploid eggs that develop into female workers or gynes (i.e., young virgin queens). Queens typically have an exceptionally large abdomen and well-developed ovaries, enabling them to lay up to two thousands of eggs per day in some species, and positioning them among the most fecund females in the animal kingdom (Wilson, 1971).

Honey bee queens are remarkably fecund, with reports of individual queens laying as many as 2000 eggs in a single day (Laidlaw and Page, 1997). This fecundity is possible because the queen is constantly tended by workers who prepare egg cells, feed her with a rich royal jelly diet, and care for all her needs. Here, we tested the hypothesis that one of the mechanisms enabling the queen to achieve such remarkable fecundity is her ability to lay eggs over many hours during the day. Earlier human-based observations suggesting that queens are active around the clock with no diurnal rhythmicity lend credence to this hypothesis (Johnson et al., 2010).

The temporal activity of egg-laying queens differs from the diurnal pattern of gynes, which presumably allows them to precisely time their nuptial flight. Thus, the honey bee queen changes her activity rhythms along with her reproductive state (Laidlaw and Page, 1997). Somewhat different findings were reported by Harano et al. (2007) who monitored individually isolated virgin gynes and mated queens and found that both showed circadian rhythms in locomotor activity. Their study suggests that both mated and virgin queens have a fully functional circadian clock system. Similar plasticity in activity rhythms is also found in bumble bee queens, which switch between activity with and without circadian rhythms according to their age, reproductive state, and the presence of brood (Eban-Rothschild et al., 2011).

Tracking queen egg laying is challenging for several reasons. First, the honey bee queen lays her eggs in the dark cavity of a nest densely populated with thousands of bees. Second, mated queens have negative phototaxis suggesting that illuminating them for recording purposes may affect their behavior (Richard et al., 2007). Finally, the eggs are laid in the bottom of elongated opaque wax cells, making it difficult to determine whether an egg was laid without interrupting the natural behavior of the colony. Human observer recordings of queen egg-laying behavior in colonies suggested that they lay eggs around the

clock (Johnson et al., 2010), but the presence of eggs was not recorded in this study. Human observer-based focal sampling observations are also limited in their ability to assess egg-laying rhythms over several circadian cycles.

To contend with these challenges, we developed novel protocols to continuously monitor queen activity and egg laying for several consecutive days. First, we adapted a barcode-based monitoring system for precise high-resolution tracking of queens in a small observation hive with access to free foraging outside. Using this system, we found that queens are active around the clock with no circadian rhythms, supporting and extending earlier behavioral observation studies. We also developed a new laboratory system to continuously and precisely record the number of eggs laid in small transparent artificial combs. Using this system, we show that honey bee queens lay eggs throughout the whole day and that the illumination regime influences overall fecundity as well as the temporal organization of egg laying.

MATERIAL AND METHODS

Experiment 1. Queen Locomotor Activity in Observation Hives

We reanalyzed existing data of queens from colonies in which all the bees were tagged with an individual barcode and were continuously recorded at 1 picture per second with a computer-controlled high-resolution camera connected (Gernat et al., 2018). In the current study, we used the location of queens to calculate their locomotor activity over time. The original data were recorded at the University of Illinois at Urbana-Champaign Bee Research Facility. Briefly, newly emerged bees (<24-h-old) and a mated queen were tagged with a 2.1×2.1 mm “bCode” barcode on the thorax and placed into a glass-walled observation hive with a 1-sided plastic honeycomb. The hive was kept indoors and connected to the outside with a plastic tube. A wire mesh between this tube and the hive exit prevented bees from leaving the hive during the first 2 days. The colony was provisioned with sufficient pollen and honey for the duration of the entire experiment. The locomotor activity of the queens recorded by the cameras was estimated using custom-made software. More specifically, distance moved was calculated as the difference between the queen’s locations in successive images and summed over consecutive 60-sec time windows. If the queen was not detected for up to 10 min, we assumed that she did not move during this time period. We tracked the activity of queens of 5 colonies over a period of at least 9 consecutive days.

Queen Egg Laying Tracking Under Controlled Conditions

We tracked the egg laying of 39 European honey bee (mix of *Apis mellifera ligustica* and *carnica* typical to Israel) queens that were purchased from a commercial breeder in Israel. The queens were 2-4 months old and were naturally mated. After mating, each queen was housed in a small beehive with ~1000 bees in which she laid her first eggs. Upon arrival to our bee research facility, each queen was placed in an egg-laying monitoring cage together with 5g of 1-day-old worker bees (approximately 50 bees) that had emerged from honeycomb frames at the Edmond Safra Campus of the Hebrew University of Jerusalem, Jerusalem, Israel. The workers used in each experiment were obtained from 2 to 3 colonies and were mixed and randomly assigned to cages to minimize genetic variability between cages. Each experimental cage was provisioned with a 5-ml tube full of honey, a tube with water, a tube with sugar syrup (30% weight/volume), a pollen paste (70% grain pollen and 30% sugar syrup), and a piece of a honeycomb (about 6 cells) filled with fresh bee bread collected from the colonies. All food was provided ad libitum and replaced every other day. The cages with a queen and workers were kept for the first 3 days in an incubator ($34^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$, relative humidity [RH]% = $65\% \pm 5\%$) and were monitored once a day. Queens that failed to consistently lay eggs (about 20% of the queens) were assumed not adapted to our monitoring cages and were excluded from the study.

Egg-laying Monitoring System

Our egg-laying monitoring system includes a Queen Monitoring Cage (Fine et al., 2018) equipped with a hexagonal plate made of clear plastic, resembling in shape a regular honey bee wax comb. The transparent cage and plastic frame enable us to clearly see and photograph the eggs in each cell at a single egg resolution. The cages were placed in an environmental chamber ($34^{\circ}\text{C} \pm 1^{\circ}\text{C}$, RH% = $50\% \pm 10\%$) and kept under constant dim red light (mean wavelength = 660 ± 10 nm) that the bees cannot see well. A surveillance camera (Hikvision DS-2CD2120F-IS) was placed at a distance of 30 cm from the cage front and was directed to the back of the cage (Figure 1a). A picture of the transparent egg-laying plate was recorded every 10 min and automatically saved on the camera's memory card. The egg-laying plates were replaced every 10-24 h making sure that the queens had a sufficient number of empty cells in which they can lay eggs. Using this procedure, we followed all eggs laid by the queen for over a week. Egg plate replacement and food provisioning (every

other day) were performed at different hours during the day to avoid entraining the circadian rhythms of the queens and workers to these events.

In each experiment, we used 4 cameras, each one tracking a single cage. Given that circadian rhythms in honey bees can be synchronized by substrate-borne vibrations (Siehler and Bloch, 2020; Siehler et al., 2021), we placed each cage on a piece of vibration-absorbing rubber foam. The timing of egg laying was not statistically correlated for any pair of queens in the same trial, consistent with the premise that adjacent cages did not influence each other egg-laying rhythms. Altogether we performed twelve trials, each lasting 7-14 days, under various illumination regimes (see details below).

We validated the accuracy of the egg tracking system by comparing photo records and manual counting of the number of eggs on the same plate. This comparison detected small inconsistencies of less than 3% difference between the manual and picture-based egg counting. The reasons for these inconsistencies were: 1) in some cases, queens laid more than 1 egg in a cell, which was difficult to detect by the camera but was detected during manual counting. 2) In other cases, eggs were scavenged by the workers and were therefore missing in the manual counting. Given the scarcity of these events, the number of eggs recorded using the 2 counting methods was highly correlated (Figure 1b; Pearson correlation $R^2=0.99$; $p \ll 0.001$, $n=31$ counts).

The Influence of Illumination Regime on Egg-laying Rhythms, General Experimental Design

In the first 3 days, the cages were kept in an incubator ($34^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$, RH% = $70\% \pm 5\%$), allowing the workers to mature and adjust to the cage, and for the queen to acclimate and start laying eggs. The cages were then transferred to the monitoring room in which their egg laying was recorded under various illumination regimes (Figure 1c shows a schematic illustration of the experiment design). We performed 3 experiments in which we monitored egg laying under different illumination regimes:

Experiment 2. Queen Egg Laying Under Constant Darkness (DD). The bees were monitored under constant dim red light (9 Lux, wavelength = 660 ± 10 nm), which bees cannot see well (Frisch, 1967). These conditions are most similar to those experienced by queens in typical hives. We tracked 15 queens under DD conditions for 7-12 days.

Experiment 3. Queen Egg Laying Under Light-Dark Illumination Regime (LD). The bees in this experiment experienced 12 h of white light (490 Lux) from 0500 to

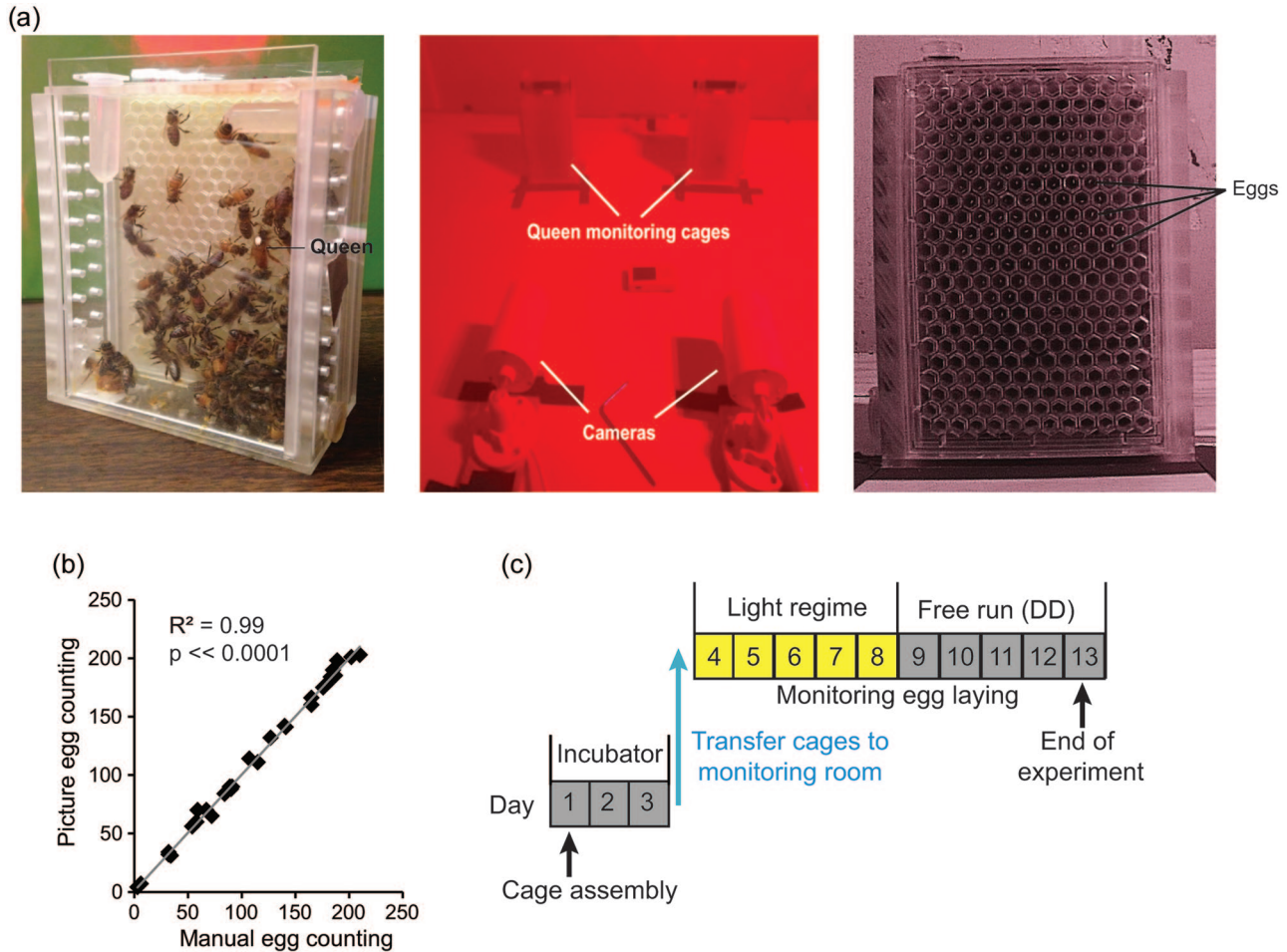


Figure 1. Tracking egg-laying of honey bee queens. (A) The cage with artificial honeycomb. Left panel: A honey bee queen monitoring cage (QMC) housing a fertile queen (marked with a white dot) and ~50 workers. Middle panel: An egg tracking system with two QMCs and two surveillance cameras. Right panel: A representative photo taken by the surveillance camera. Individual eggs can be seen in cells in the middle part of the egg-laying plate. (B) The correlation between image analyses based on egg counting from photos recorded by our time-lapse video system (Y-axis) and manual counting by a human observer analyzing the same egg-laying plate (X-axis). Each black square represents the number of eggs counted in one day ($n=31$). The grey line depicts the linear regression model for the relationship between image analysis and manual egg counting. The R^2 and the p -value summarize the results of a Pearson correlation analysis. (C) Schematic description of the general experimental outline in Experiments 2-4 testing the influence of the illumination regime on egg-laying. The numbers represent the days of the experiment. White-filled boxes – days in which we exposed the bees to the tested illumination regime (“Light regime”); grey-filled boxes - the later part of the experiment during which the cages were released to constant darkness (“Free run (DD)”).

1700 h, and the other 12 h of the day under dim red light (as in the DD conditions). After 5-7 days under the LD illumination regime, the bees were released to constant DD for an additional 4 days in which their rhythms could free run. We tracked 13 queens under LD conditions, 2 of them were tracked only under the LD conditions without a DD phase (Supp. Fig. S4)

Experiment 4. Queen Egg Laying Under Constant Light (LL). The cages with the bees were kept in constant light for the first 6 days of the experiment and then the illumination regime was changed to DD (dim red light as in the DD conditions) for the remaining 4 days of the experiment. Three queens did not lay any eggs

during the whole experiment (including the free-running DD period) and were excluded from the analyses. We analyzed the egg laying of 11 queens under LL conditions, all laid eggs during the free-run DD period. In 4 out of the eleven queens, we did not track the egg laying at the DD period besides confirming that the queen did lay normally (Supp. Fig. S3).

Egg-laying Rhythms, Data Analysis

The number of eggs in the plate was counted from pictures recorded every hour (Movie S6) using ImageJ (NIH). For each hour, the number of new

eggs added since the previous hour was recorded in an Excel table with the time of day and the number of eggs laid in the preceding hour. This produced 24 data points per day for at least 5 consecutive days. To assess overall fecundity, we compared the number of eggs per hour within and between experiments using a 1-way ANOVA between experiments, or a paired *t*-test for the same queens under different conditions. These analyses were done using SPSS (IBM).

Circadian Rhythm Analysis

The activity data of the observation hive queens were imported into the Clocklab software package. We analyzed the data using the Clocklab software with 10 min bins, using χ^2 periodogram analysis. The queen was defined as stationary if she did not change her position for an entire 1 min (with error = 1 tag size).

To analyze circadian rhythms in egg laying, we exported the Excel sheet data and arranged it in a format that can be imported into the Clocklab software package in which we analyzed these data. We used χ^2 periodogram analysis with 60 min bins to determine whether the rhythms are statistically significant and the free-running period (FRP) of the rhythm. We also generated actograms, a visual representation of the circadian egg-laying rhythm. A queen was considered to have a statistically significant circadian rhythm if the periodogram peak was higher than the $p=0.001$ significance threshold line and had a FRP in the range between 22-26 h. We used the Oriana 2.0 circular statistics software (Rockware) to analyze and present phase synchronization data. The figures were drawn using Excel or Oriana and prepared for publication using Adobe Illustrator.

RESULTS

Queen Locomotor Activity in Observation Hives

The queens of the 5 colonies monitored in Experiment 1 were active around the clock with almost no records of periods with no movement during the entire monitoring session. The queens were active most of the day with only $3.66 \pm 0.69\%$ of the time in which they did not show any movement (Figure 2). Queens of 3 out of the 5 tracked colonies showed circadian rhythms in locomotor activity in the first few days but lost these rhythms in the later part of the experiment (Figure 2). The other two queens did not show clear circadian rhythms during the entire experiment. The stationary (motionless behavior) time that the queens spent every day

increased during the first days following the day in which the hive was opened for foraging. The overall activity of the queens was reduced and the pattern was in general similar from day 5 until the end of the experiment (Suppl. Fig. S2).

The Influence of Illumination Regime on the Total Number of Eggs Laid

To have a general assessment of the influence of the illumination regime on queen fecundity, we compared the number of eggs laid by queens under the 3 illumination regimes tested in Experiments 2-4. The illumination regime had a significant effect on the number of eggs laid by the queens (Figure 3a and 3b, 1-way ANOVA, $F=13.4$, $p<0.001$). Queens laid on average 4.30 ± 0.55 eggs/h (103.7 ± 13.2 eggs/day) under DD (Experiment 2), 3.94 ± 0.36 eggs/h (94.6 ± 8.7 eggs/day) under LD (Experiment 3), and only 0.99 ± 0.46 eggs/h (23.8 ± 16.3 eggs/day) under LL (Experiment 4). The number of eggs laid under LL was significantly lower than under the two other illumination regimes (Tukey post hoc test, $p=0.001$, for both LL vs DD and LL vs LD). These differences disappeared when the queens were released from the experimental conditions to free-run under DD conditions (Figure 3a and 3b; 1-way ANOVA, $F=0.76$, $p=0.47$). The number of eggs laid/h in Experiment 3 was not changed when released from LD to DD illumination regime time (paired *t*-test, $p=0.176$). By contrast, the queens in Experiment 4 laid significantly more eggs in the later part of the DD illumination regime compared to the first session in LL (paired *t*-test, $p<0.001$).

When we looked more closely at the frequency distribution of eggs laid per hour in the 3 experiments we found that queens in DD laid at least 1 egg/h during 59% of the day and more than 10 eggs/h during 14.6% of the day (Figure 3a). Under LD illumination regime, queens laid eggs for 50% of the day and more than 10 eggs/h for 14.2% of the day (Figure 3a). The frequency distribution of the number of eggs laid per hour differed between the LD and DD conditions (Z-test for proportion $Z=3.76$ $p<0.01$). Under LL, the queens laid during only 14.5% of the day and more than 10 eggs per hour at only 2.8% of the time. Egg laying in LL illumination regime was significantly lower compared to the DD or LD illumination regimes (Figure 3a, Z-test for proportion LL vs DD: $Z=21.6$ $p<0.001$, LL vs LD: $Z=18.0$, $p<0.001$). When the queens in Experiment 3 were transferred from LD to DD illumination regimes, they laid during 56% of the time, not different from their egg laying during the LD session (Figure 3a, Z-test for proportion $Z=1.74$ $p>0.05$) or

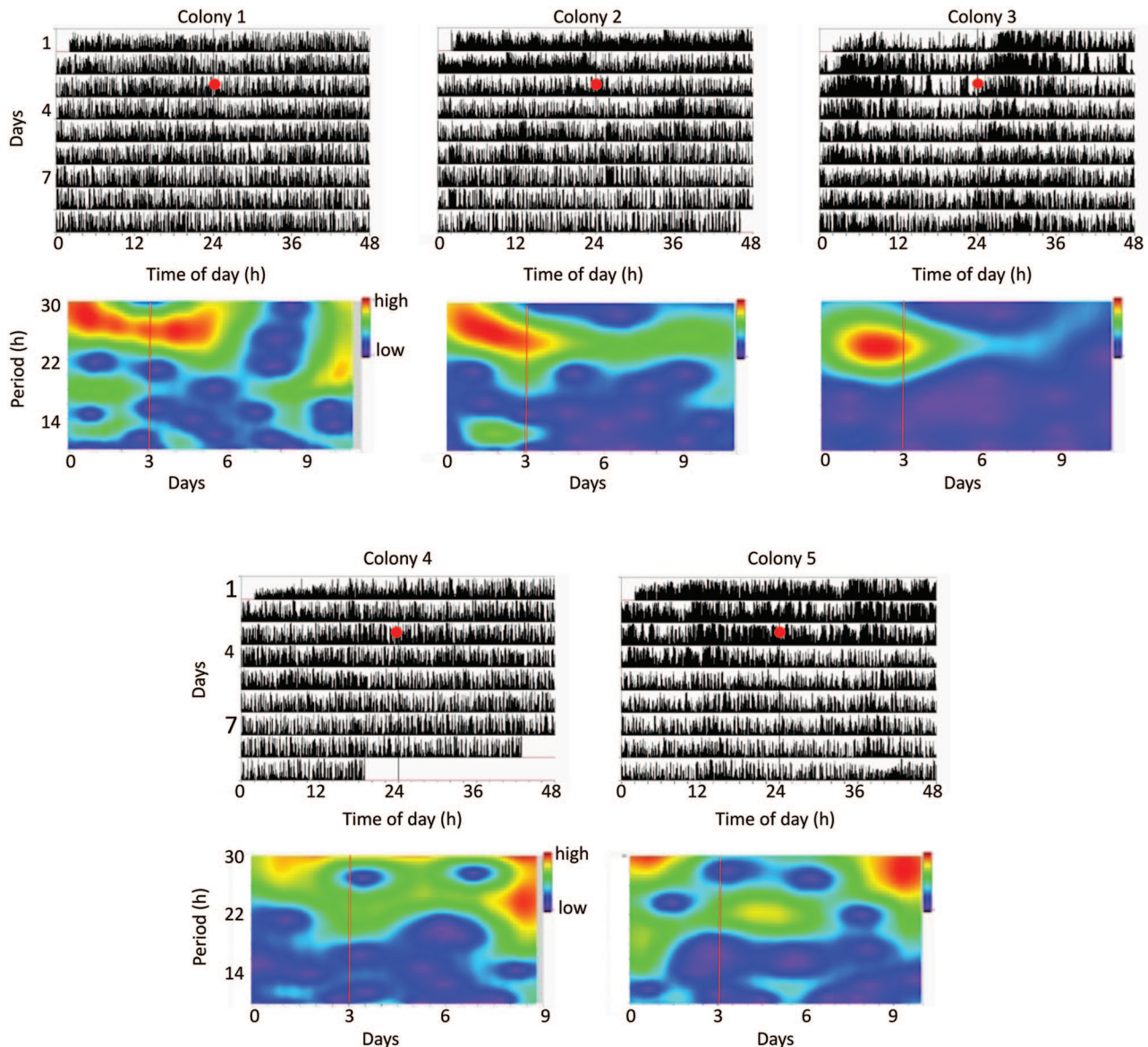


Figure 2. Locomotor activity of queen honey bees in small colonies with access to the outside. Data for five queens from different colonies are shown. The upper plot for each queen shows a double-plot actogram in which the X-axis depicts the time of the day, and the Y-axis the days of the experiment. The heights of bars within each day show the locomotor activity in 5-min bin intervals. The lower panel for each queen shows wavelets across the days of the experiment (X-axis); the color shading corresponds to the strength of rhythms for each tested period. Red circles in the actograms and lines in the wavelet plot depict the time in which the hive entrance was opened to the outside.

from the DD queens (Figure 3b, Z-test for proportion $Z=1.63$, $p>0.05$). When the queens in Experiment 4 were transferred from LL to DD, they significantly increased their egg laying and laid during 49% of the hours (Figure 3b, Z-test for proportion $Z=11.8$, $p<0.001$), which is not different from queens monitored under DD in Experiment 2 (Figure 3b, Z-test for proportion $Z=2.55$, $p>0.05$).

Under DD illumination regime the queens in Experiment 2 laid 56% of the eggs during the

subjective night (referred to as the time between 0500 and 1700 h). The pattern during the LD stage in Experiment 3 was the opposite with 57% of the eggs laid during the light hours (0500-1700 h, which are also the relative daytime, Figure 3c, Two samples proportion test, $Z=17.4$, $p<0.001$). This comparison however should be taken with caution because the DD queens in Experiment 2 were not entrained by a similar LD regime before released to DD conditions.

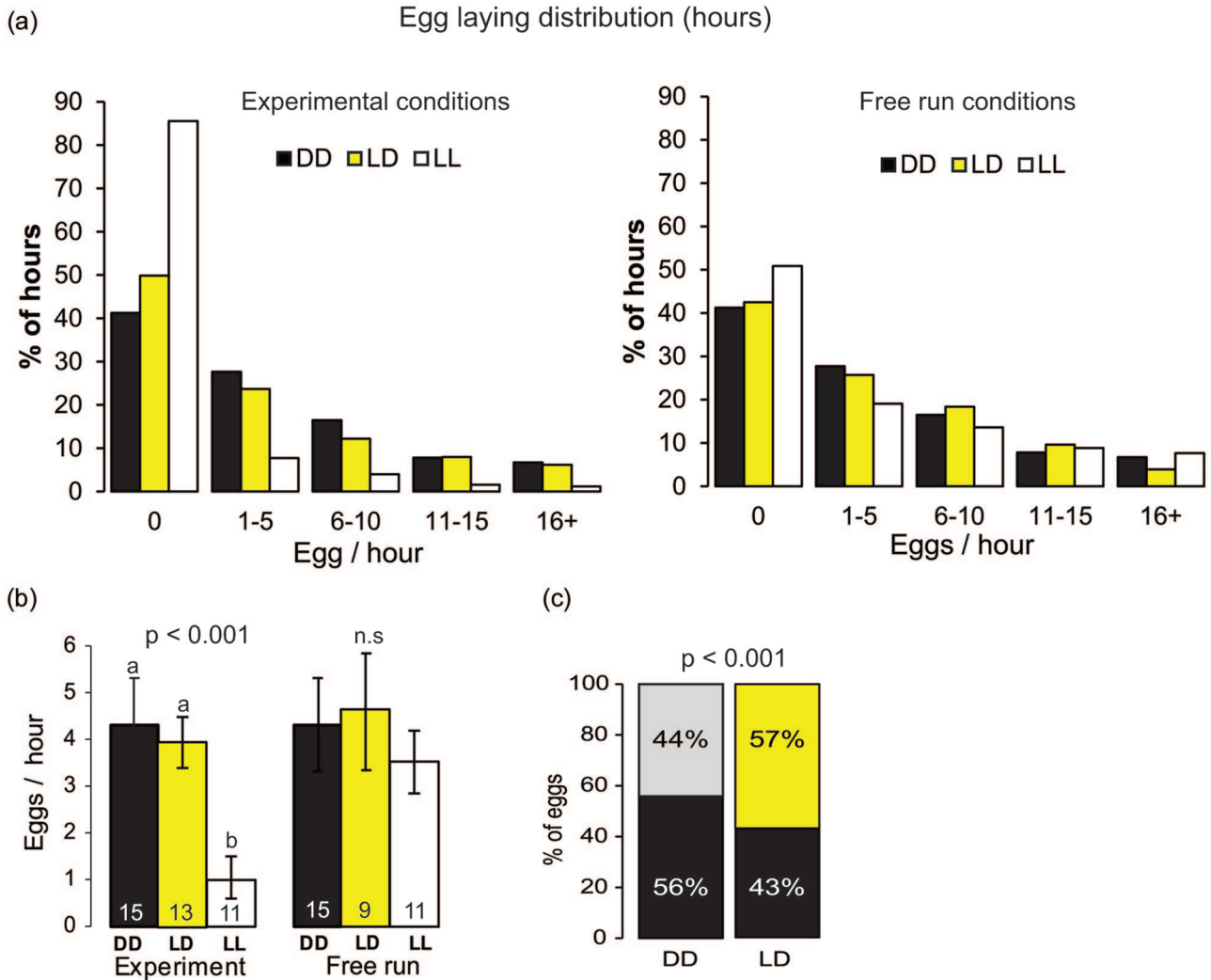


Figure 3. The influence of the illumination regime on queen fecundity. (A-left) Histogram summaries of the frequency distribution of egg-laying under different illumination regimes, and (A-right) during the last part of the experiment in which the bees were released to constant darkness conditions. (B-left) Summary of queen fecundity under the three different illumination regimes, and (B-right) after released to constant conditions. Each bar shows the mean \pm SE. Black – constant darkness (DD); gray – 12 hrs. light: 12 hrs. dark illumination regime (LD); white – constant light (LL). The p -values are from a one-way ANOVA and bars marked with different small letters are significantly different in a Tukey posthoc test; the sample size is shown at the bottom of each bar. (C) The percentage of eggs laid during the subjective day (grey) and subjective night (black) under DD conditions and light (white) and dark (black) under the LD condition. The p -value is from a z-test for proportions.

The Influence of Illumination Regime on the Distribution of Egg Laying During the Day

Queens under DD typically laid eggs around the clock (Figure 4a, left panel; Suppl. Fig. S3) and only 3 out of 15 had a circadian rhythm (i.e., with a period of 22-26h; Figure 4b; Suppl. Fig. S3 in which the actograms are sorted in descending strength of circadian rhythm) in egg laying. Ten out of thirteen queens laying under the LD illumination regime had strong 24h rhythms in egg laying. The daily onset of egg laying was synchronized among queens (Figure 4c, Rayleigh

test, $Z=3.35$, $p=0.03$, $n=13$) but the phase did not show clear alignment with the time of light on. When the queens were transferred from LD to DD, all those that showed daily rhythms in LD kept it during the free-run DD session which lasted until the end of the experiment. However, the onset of the egg laying was not synchronized among queens (Figure 4c, Rayleigh test, $Z=1.6$, $p=0.202$, $n=10$). Only 3 of the 12 queens in LL laid a sufficient number of eggs to allow rhythm analyses. Two of these queens had a circadian rhythm in egg laying. When the queens in this experiment were transferred from LL to DD illumination regime

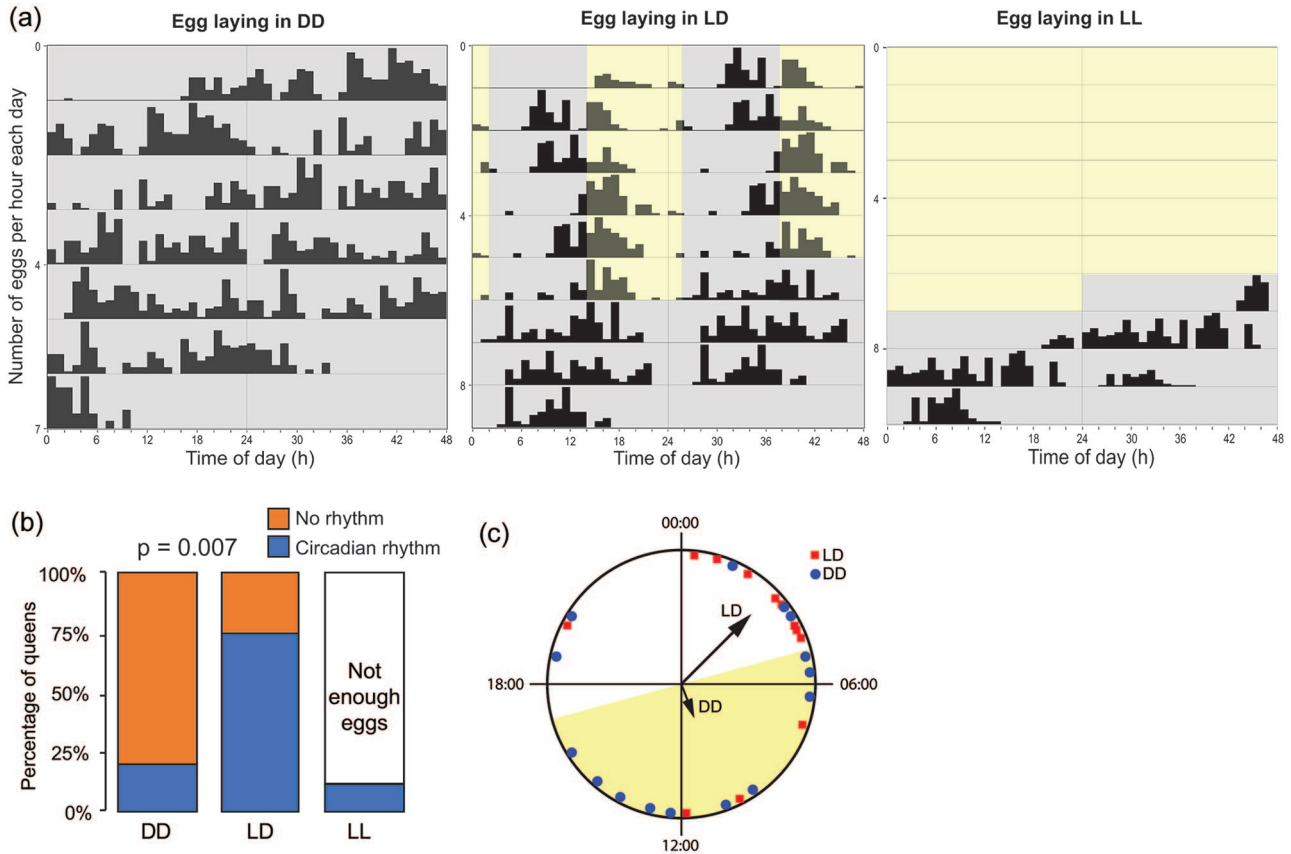


Figure 4. The influence of the illumination regime and the temporal organization of egg-laying. (A) Representative actograms of queen egg-laying under the three tested illumination regimes. The numbers on the Y-axis represent the day of the experiment. The height of the black bars within each day corresponds to the number of eggs laid during one hour. Data for each day are double plotted to facilitate visual detection of rhythmicity. (B) Percentage of queens with a statistically significant egg-laying rhythm with a period of 22–26 hours under different light conditions (DD, $n = 15$; LD, $n = 13$; LL, $n = 11$). The P -value summarizes a 2x2 Fisher Exact test comparing the DD and the LD conditions (The queens in LL did not lay enough eggs to be included in the analysis). (C) The time of the daily onset of egg-laying for queens during the LD phase (white squares) and free-run DD phase (black dots). The black arrow depicts the Rayleigh vector which points to the average phase and its length corresponds to the degree of synchronization ($* = p < 0.05$). The gray shading depicts the period of light under LD.

and started to lay eggs (Figure 4a; Suppl. Fig. S5), all beside one, which also showed laying rhythms in LL, did not have a circadian rhythm.

DISCUSSION

The honey bee queen can lay up to 2000 eggs per day (Laidlaw and Page, 1997), which is substantially higher than all known solitary or primitively eusocial bees. Their remarkable fecundity is thought to reflect an evolutionary selection for increased fertility allowing a single mated female to produce massive families of up to more than 50,000 daughter bees. But how can a single female insect lay such a large number of eggs, which means an average of an egg laid every 2 min, or even more? Does she lay eggs around the clock without resting at all or is her egg laying limited to certain parts of the day, freeing up some of her time to rest and recover?

To answer these questions, we first reanalyzed data from a barcode-based tracking system that enables tracking the location of a queen in a colony with a higher level of resolution than in previous studies (Gernat et al., 2018). Our records show that honey bee queens are active around the clock with no, or with only short periods of, inactivity. Our activity traces show that the honey bee queen moves almost constantly and is stationary merely 3.66% of the time, which is much lower than the 30% estimated based on human observations (Johnson et al., 2010). We assume that this difference reflects the higher sensitivity of the automatic monitoring system which detects small movements that are typically not recorded by human observers.

Queens showed no circadian locomotor activity rhythm during all or most of the tracking sessions. For queens that did show periods with circadian rhythms, they were limited to the first few days of the experiment. Perhaps the initial period of circadian

rhythmicity reflects a transitional state from an earlier stage in which young queens show rhythms and rely on their circadian clock to time their mating flights and return to the hive (Koeniger and Koeniger, 2000). It is also possible that the initial rhythm reflects an influence of our experimental procedure; we established the colonies with newly emerged bees and opened the hive entrance only on the third day. Thus, it is possible that the callow workers could not fully support the queens during the first days of the experiment and therefore some of the queens did not start egg laying, which is thought to induce around-the-clock activity (see below). Our high-resolution monitoring of queens in colonies supports and extends earlier studies in which queen activity was monitored by means of human observations and indicates that egg-laying queens are typically active around the clock with attenuated circadian rhythms (Harano et al., 2007; Johnson et al., 2010).

Although honey bee nests are dark and tightly thermoregulated (Winston, 1987), it is hard to believe that queen activity around the clock is a simple reflection of a constant environment. Foragers fly in and out of the nest during the day and sleep inside the nest during the night, creating oscillations in the nest microenvironment. Surrogates of forager activity such as substrate-borne vibrations and nest volatiles are sufficiently strong to entrain the circadian clocks of nest bees (Siehler et al., 2021; Siehler and Bloch, 2020), even under a conflicting LD illumination regime, indicating that social time givers can override photic entrainment (Beer et al., 2016; Fuchikawa et al., 2016; Siehler et al., 2021). Similarly, around-the-clock activity was also recorded for egg-laying bumble bee queens which have significantly lower fecundity (Eban-Rothschild et al., 2011). Taken together, these studies suggest that the queen is active around the clock because her reproductive state influences her activity rhythms. It should be also noted that honey bee queens are constantly tended and nourished by worker bees, which may also affect their level of activity.

The queens monitored with our new laboratory-based egg-laying tracking system (Experiments 2-4) laid on average about 103 eggs per day, and up to a maximum of 283 eggs per day, which is comparable to queen fecundity in previous studies using the same system (Fine et al., 2018; Fine, 2020; Fine et al., 2021). These numbers are lower than what occurs in typical-sized field colonies (Laidlaw and Page, 1997). The number of eggs laid by a queen in a colony is strongly affected by the number of workers, which determines colony efficiency in regulating the hive microenvironment and providing proper queen nourishment (Winston, 1987). For example, in an observation colony with 1500 or 3000 workers, queens lay about 300

eggs a day (Wu-Smart and Spivak, 2016). In our system, the queens were accompanied by only about 50 workers, which can account for the relatively low fecundity. The lab setup and artificial comb material may also have negatively affected fecundity. The relatively lower fecundity in our lab assay could enable the queen to restrict egg laying to only a portion of the day. However, under constant-dark conditions (Experiment 2), which are most similar to the dark cavity of the honey bee nest, most of the queens laid eggs around the clock with no circadian rhythms. This absence of circadian rhythms in egg-laying behavior is consistent with the lack of circadian rhythms in locomotor activity of queens in larger colonies (Figure 1; Johnson et al., 2010).

Taken together, these observations are consistent with the hypothesis that the queen reproductive state influences her circadian system. This hypothesis is consistent with studies with bumble bees in which the queen switched between activity without or with circadian rhythms based on whether brood was present or not, respectively (Eban-Rothschild et al., 2011). Importantly, some of the bumble bee queens for which the brood was lost or removed switched to activity around the clock with no circadian rhythms before resuming egg laying, suggesting that their activity rhythms were regulated endogenously by their physiological state and not merely by the need to tend brood around the clock (Eban-Rothschild et al., 2011). Around-the-clock active bumble bee queens lay up to a few dozen eggs per day, even less than honey bee queens in our experimental system. It should be noted that by contrast to bumble bee queens, honey bee queens do not care for their offspring. Therefore, their lack of circadian rhythms cannot be explained by a need to tend the brood.

Constant light had a strong negative effect on egg laying; most queens ceased egg laying under LL light regime (Experiment 4; Figures 2 and 3). This finding is not surprising given that honey bee queens move away from light (negative phototaxis) and find continuous exposure to light stressful (Richard et al., 2007). Evidence also suggests that light compromises some worker behaviors (Morse, 1965; Moore and Rankin, 1985). Given these adverse effects of light, it was unexpected to note that under periodic exposure to light (LD, Experiment 3), queen fecundity was overall not lower than in DD. Moreover, most queens showed diurnal rhythms in egg laying and surprisingly, laid more eggs during the light phase. Why most queens did not lay eggs in LL but did lay during the light phase of the LD regime is not clear. Perhaps the stress associated with constant light, but not with a 12:12 LD regime, is high enough to compromise oogenesis or block the laying of mature eggs.

Given that honey bee queens depend on the workers to prepare the cells and take care of her, the regulation of egg laying is influenced by both the queen and the workers. Worker bees are typically diurnal with higher activity during the light phase. Even nurse bees, which care for the brood around the clock in colonies under LD illumination regime, do have functional clocks that can be entrained by light (Bloch, 2010; Eban-Rothschild et al., 2012; Fuchikawa et al., 2017; Beer et al., 2018) and show rhythms in some behavioral and physiological processes (Nagari et al., 2017a, 2017b). Thus, perhaps some worker rhythms influenced the queens to lay during the light phase of the LD illumination regime. However, it should be noted that most queens started to lay eggs during the dark phase of the LD illumination regime (Figure 4a and 4c; Suppl. Fig. S4). Thus, it is possible that after starting to lay eggs, it takes some time until they respond to the light and cease egg laying. Additional studies are needed to understand the complex influences of the illumination regime on honey bee egg laying and the possible contribution of the workers to the expression of these rhythms.


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CONFLICT OF INTEREST STATEMENT

The author(s) have no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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NOTE

Supplementary material is available for this article online.

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