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Honey Bee Queens Do Not Count Mates to Assess their Mating Success

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Abstract The mating system of honey bees (genus *Apis*) is extremely polyandrous, where reproductive females (queens) typically mate with 12 or more males (drones) during their mating flight(s). The evolutionary implications for hyperpolyandry have been subject to considerable debate and empirical testing because of the need to understand the proximate mechanisms that drive such extreme mating behavior despite the potential costs. The ability of queens to gauge and adjust their reproductive success is therefore important for selection to act on queen mating number at both the evolutionary (colony-level) and proximate (individual-level) timescales. We observed the mating flight activities of 80 queens in their respective mating nucleus hives each with a modified entrance that restricts flight attempts. We also attached a small weight (0, 16, or 38 mg) onto each queen's thorax as a means of imposing additional flight costs. We then compared queens that were restricted from taking multiple mating flights to those that started oviposition after a single flight for their mating numbers as quantified by microsatellite analyses of their respective worker offspring. We found that neither additional weight nor restricted mating attempts had any significant effect on the effective mating frequencies of the experimental queens during their single mating flight. This observation suggests that queens are not adjusting their nuptial flight activity according to their precise mating number during their flight. These findings provide insights into the proximate regulation of honey bee queen mating behavior and the fitness consequences of hyperpolyandry at the colony level.

Keywords Polyandry · mating systems · honey bee reproduction · paternity analysis · *Apis mellifera*

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Introduction

Social insects have been highly favored models for the study of sociobiology because of their often elaborate division of labor and extreme cooperative behavior such that workers allocate their tasks in a coordinated effort to maximize colony fitness. Kinship theory has also given focus to intracolonial relatedness among the workforce, where it is widely asserted that high relatedness among nestmates is a key requirement for highly cooperative behaviors to evolve. Queen number (polygyny) and mating systems (polyandry) can greatly affect the genetic structure within colonies and have therefore garnered widespread attention in the field (Boomsma and Ratnieks 1996; Nonacs 1988; Zeh and Zeh 2001).

Polyandry, when a female mates with multiple males, is a relatively rare but widespread mating system in the social Hymenoptera (Strassmann 2001), and where extreme polyandry (>5 matings) occurs it tends to be associated with large and highly advanced insect societies (Hughes et al. 2008). One of the most outstanding examples is honey bees (*Apis* spp.). The putative benefits of such hyperpolyandry in honey bees are many and not mutually exclusive (reviewed in (Palmer and Oldroyd 2000), and the 'genetic variance' hypotheses are widely regarded as the most plausible evolutionary explanations (e.g., (Crozier and Fjerdingstad 2001).

The proximate regulation of multiple mating, however, has drawn less attention than its adaptive consequences in social insects. Nonetheless, the manner by which queens acquire their mates is critical to understand how the behavior has evolved and the process by which selection may act; neglecting the behavioral process and focusing solely on its outcome (i.e., average intracolony genetic relatedness) disregards the important behavioral mechanisms upon which natural selection acts to favor polyandry. Queen honey bees (*A. mellifera*) are highly and nearly ubiquitously polyandrous, mating with an average of ~12 drones on 1–5 mating flights early in their lives (Ruttner 1956; Woyke 1962). A queen mates with multiple males in rapid succession on each flight, and she returns back to her nest after ~10–30 (or more) minutes with her median- and lateral oviducts swollen with up to 20 μl of semen from her multiple mates. Only 5–10% of each mate's sperm actively migrate up into a queen's spermatheca (Oldroyd et al. 1998), with the surplus semen being expelled from her sting chamber. Once a queen begins egg laying, she will no longer engage in mating behavior for the remainder of her lifespan, which can last upwards of 2–3 years.

Descriptive studies of honey bee mating abound (e.g., (Gary 1963; Koeniger 1988; Lensky and Demter 1985; Roberts 1944; Ruttner 1956), and many others have investigated the mechanisms that prompt queen oviposition, including some elegant studies testing the physical stimulus of copulation (Koeniger 1976; Koeniger 1981), carbon dioxide exposure (Mackensen 1947; Nino et al. 2011), and the total insemination volume of semen deposited in the oviducts (Kocher et al. 2009; Kocher et al. 2010; Nino et al. 2013b). However, relatively few studies have investigated the potential behavioral regulation of mating number in queen bees, and their findings are equivocal. Tarpy and Page Jr (2000) compared the mating numbers of queens that were prevented from taking second mating flights (incomplete mating) to those that started oviposition after their mating flight (complete mating). They found little to no difference in mating frequency between the two groups, and they concluded that queens do not exhibit strong behavioral control over mating number. Following the same basic experimental

design, Schluns and colleagues (2005) did detect a significant difference in mating number between queens that had either complete or incomplete mating, leading them to conclude that natural selection has optimized queen mating behavior by minimizing their nuptial flights while maximizing mating number. Moreover, Koeniger and Koeniger (2007) found a weak negative correlation between flight time and sperm count, arguing that this indicates that queens actively monitor their mating success during flight. Using a different approach, Hayworth and colleagues (2009) added multiple artificial weights to queens during their mating flights and showed a decrease in flight number and lower mating frequencies with increasing flight costs, suggesting that queens adjust their mating behavior in response to increased in-flight energetic costs.

Given the equivocal findings in the literature, it is therefore important to elucidate how individual queens may regulate their mating behavior in light of their previous mating experience and imposed flight costs. Here, we investigated and aimed to clarify these questions of how queen bees acquire their mates by simultaneously varying both an experimentally induced flight cost (added weights) and regulated mating (flight number) in order to determine whether or not honey bee queens exert demonstrable control over their mating number. In doing so, we explored whether the results suggest regulation of polyandry at the individual level and ask whether selection for hyperpolyandry, and the increased intracolony genetic diversity that it ultimately confers, is acting more proximately at the individual queen level or on a more evolutionary scale at the colony level.

Materials and Methods

Experimental Queens and Colonies

In May 2013, we raised sister queens from a single genetic source following standard 'grafting' techniques in late April 2013 (Laidlaw Jr and Eckert 1962). We then placed individual queen cells into separate mating nucleus hives each containing three frames of brood and food (stored honey and pollen) and a population of ~1000 unrelated workers. All hives included modified entrances (Fig. 1a) each with two queen-excluder gates and a clear, Plexiglass ceiling to facilitate observations during mating flights (see Koeniger 1976; Schluns et al. 2005; Tarpy et al. 2011). The queen excluder entrances allowed the workers to forage freely, but prevented the queens from exiting their hives.

Three days after the emergence of the queens, when queens are still young and unlikely to fly, we opened each hive in the early morning (prior to when queens take mating flights) to locate and temporarily capture each queen. We randomly assigned each queen to one of three weight treatment groups: 0 mg (paint only), 16 mg, and 38 mg. The added weights were manufactured from flexible kitchen magnets using a standard hole punch, then painted to facilitate observation. We glued the weights onto the queens' thoraces using Duco Cement® to ensure adequate adhesion (Tarpy and Page Jr 2000). Thereafter, queens were returned to their respective mating nuclei and caged in JZ-BZ queen cages for ~3 h to allow ample time for the glue to cure and for the colony to re-acclimate to the queen.

Seven days after their emergence, we initiated daily observations of queen flight activity during the mid- to late-afternoons (typically 1400 to 1800 h), and ended when no more queens were engaged in mating flights (per Tarpy and Page Jr 2000). Each day, we opened

Fig. 1 **a** Modified entrances of the mating nucleus colonies with two removable gates of queen excluder material to restrict mating flights of queens while allowing normal foraging of workers. **b** A weighted queen returning from a successful mating flight, as indicated by the lodged mating sign in her bursa

the first queen-excluder gate that was nearest to the hive entrance (see Fig. 1a) on each hive to permit the queen to enter the elongated entrance, indicating her intent of taking a flight. Any queen that was observed in the runway had its second queen-excluder gate (that furthest from the entrance) removed to allow the queen to take flight. If a queen did fly, we noted the time of her departure and quickly replaced the first queen-excluder gate to temporarily prevent her re-entry. Colonies were monitored continuously by a minimum of three observers. Once a flying queen returned to her entrance and was observed in the runway, we again noted the time and whether or not she exhibited a 'mating sign' (the lodged endophallus(i) from her last 1–2 mates in her bursa, see Fig. 1b; Woyke 2011), and released her back into her colony before replacing both queen-excluder gates.

Each morning, well prior to queen flight activity, we opened all colonies with a queen that had a flight to inspect the combs for eggs. Because queens cease all mating activity once they initiate oviposition (Koeniger 1988), we surmised that any laying queen had concluded her mating activities. We continued to monitor queen flight activity for all non-laying queens, including those that had already taken a successful mating flight. We observed the entrances of any queen that had mated but not yet started to lay eggs, and if she attempted a second mating flight we did not permit her to do so, captured her at the entrance, subjected her to CO_2 narcotization for ~5 min, and returned her to her colony. CO_2 treatment has long been known to prompt queens to cease mating behavior and stimulate ovary development (Mackensen 1947; Nino et al. 2011), hence forcing them to lay eggs prematurely. This resulted in two experimental groups of queens: 1) those that had ceased mating behavior after one flight and initiated egg laying; and 2) those that had attempted additional mating flight and were CO_2 narcotized to stimulate egg-laying. Thus both groups took only one nuptial flight and had the same opportunities for mating, but the first was no longer behaviorally motivated to continue mating while the second wanted to mate again, but was not allowed.

Sampling and Analyses

Once the brood from each queen was verified to be viable female offspring (i.e., fertilized and therefore worker and not drone brood, ~3 weeks after laying), we removed pupae from each hive and stored them at −80 °C for genetic analyses. We

then extracted the DNA from 24 to 47 worker pupae from each colony (39.2 ± 5.34) using Chelex 100® resin (Walsh et al. 1991) following previously described protocols (see Simone-Finstrom et al. 2016). We then performed PCR for 4–8 microsatellite loci following (Delaney et al. 2011). Specifically, we used microsatellite loci *Am*010, *Am*043, *Am*052, *Am*059, *Am*061, *Am*098, *Am*125, and *Am*553 in two multiplexed PCR reactions. We ran the PCR products on an ABI 3730® sequencer in the Genomic Science Laboratory at NC State University and determined the marker set for each worker. Any PCR products for a given locus that were ambiguous or inconsistent were excluded from the analysis to avoid scoring errors. We analyzed the final marker sets using COLONY 1.2 (Wang 2004) then calculated the observed mating number (N_o) and effective paternity frequency (m_e) following (Nielsen et al. 2003).

Additionally at the end of the experiment, spermathecae of a subset of queens (0 mg, $n = 30$; 16 mg, $n = 11$; 38 mg $n = 8$) were dissected to count the total number of stored spermatozoa and to calculate the percentage of viable sperm. 57% of these queens had initiated egg laying after a confirmed mating flight (=Group 1). We completed live dissections to retrieve the queen's spermatheca following previously used methods (Delaney et al. 2011; Schluns et al. 2005), and we analyzed the sperm number and viability using a Vision CBA Analysis System® (Nexcelom Bioscience, Lawrence MA), a device that uses high sensitivity fluorescence and brightfield cell counting of dead and living sperm simultaneously, following (Collins and Donoghue 1999) by staining the sperm with Sybr 14 and propidium iodide. However, because these queens were destructively sampled in October at the end of the research season (~26 weeks after egg-laying initiated), we did not expect that they would have a full compliment of stored sperm in their spermathecae.

Analyses

Observed mating number (N_o) and effective paternity frequency among the workers (m_e) were analyzed using a two-way ANOVA with flight treatment (attempted a second mating flight or did not) and weight (0 mg, 16 mg, and 38 mg) as fixed independent variables. All statistics were analyzed with JMP Pro v10.0 (SAS, Cary NC) and are reported as mean ± SEM and with $\alpha = 0.05$, unless otherwise noted.

Results

We observed a total of 80 queens for a total of 190 individual flights (2.4 ± 0.15 SE flights per queen), which included orientation flight(s) plus the single mating flight. Orientation flights were deemed those less than 5 min in duration, while all confirmed mating flights took at minimum 12 min but up to 47 min. Of these, 68 were verified mating flights indicated by the presence of mating signs, as some queens either failed to mate or did not return from their mating flight. The average duration of all observed flights was 19.8 ± 1.0 SE min and 22.8 ± 1.0 SE min for known mating flights. Most of the queens (65%, $n = 52$) initiated oviposition following their first (and only) successful mating flight, so that about one-third (35%, $n = 28$) of the queens attempted a second mating flight the subsequent day but were prevented from doing so and compelled to lay eggs using CO_2 narcosis (see above). Many of the queens in the 38 mg treatment

group were unable to fly, and thus we removed their weights and provided them with a paint mark (thus placing them into the 0 mg treatment group). In the final measured population, 67.5% ($n = 54$) of the queens had no additional weight, 21.25% ($n = 17$) had 16 mg, and 11.25% ($n = 9$) had 38 mg (refer to Fig. 2 for sample sizes per mating groupings).

There was a wide range in the number of mates among the queens (8–25 drones), with the average observed mate number (N_o) being 13.5 ± 3.46 SE drones. The average effective paternity frequency among the workers (m_e) was 11.0 ± 5.70 SE (range 3.6–30.9). These levels of polyandry are well within the expected range for the species (Tarpy et al. 2004). There were no significant differences in mating frequency with respect to flight attempts ($F_{1,78} = 0.63$, $p = 0.43$) or added weight ($F_{2,77} = 0.53$, $p = 0.59$; Fig. 2). While numerically those queens attempting second mating flights (10.3 ± 1.10 SE) were slightly lower than those laying eggs after only one flight (11.4 ± 0.79 SE), the difference was not statistically significant. There was also a numerical trend (in the opposite predicted direction) for increased weight, where queens with no added weight had lower mating numbers (10.6 ± 0.78) than those with 16 mg (11.4 ± 1.40 SE) and 38 mg (12.7 ± 1.91 SE), but again this difference was not statistically significant.

The overall average total number of stored spermatozoa in the analyzed queens was 1,045266 ± 174,343 SE sperm with 90.9 ± 1.3% SE viability (see Table 1 for group-based data). While a fully mated queen typically stores 5–7 million sperm (Woyke 1962), we should note that these queens were partially restricted for their mating flights and analyzed for their sperm stores after a full egg-laying season (~26 weeks) in full-sized colonies, thus the lower sperm counts are indeed expected. More importantly, we did not find a difference across weight treatments ($F_{2,45} = 1.00$, $p = 0.37$) or between queens that attempted flights after a confirmed mating flight ($F_{1,45} = 0.75$, $p = 0.39$). There was also no correlation between effective paternity frequency and stored sperm number ($r^2 = 0.004$, df = 47, $p = 0.65$), unlike the positive but non-linear relationships found in previous studies (Delaney et al. 2011; Schluns et al. 2005), again likely a consequence of testing queens that were not newly mated and had significantly lowered total sperm counts.

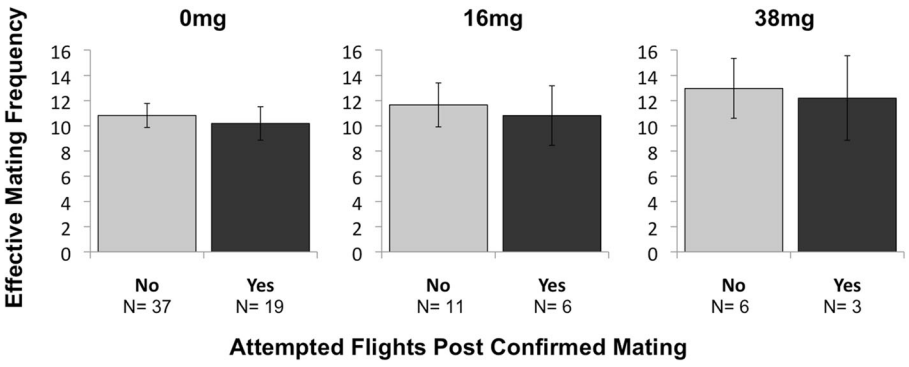

Fig. 2 Mating numbers of experimental queens. The effective paternity frequency of queens with 0, 16, and 38 mg weights were not significantly different between those that started laying after one mating flight (complete mating) and attempted subsequent flights (incomplete mating)

Table 1 Sperm count and viability. Data shown as mean ± SE for sperm counts and percent viability as determined from queens in October 2013 at the end of the field season

	Initiated oviposition after first mating flight			Attempted multiple mating flights		
Weight treatment	Total sperm count	Percent viable sperm	N	Total sperm count	Percent viable sperm	N
0 mg	1,016,192 ± 139,481	92.7 ± 1.4%	19	783,678 ± 138,571	92.4 ± 2.3%	14
16 mg	663,933 ± 190,743	85.3 ± 5.3%	6	774,183 ± 214,164	92.2 ± 1.4%	6
39 mg	663,900 ± 235,554	80.5 ± 9.2%	5	705,700 ± 22,779	93.7 ± 0.3%	3

There were no significant differences due to weight treatment or queens that initiated oviposition after one flight versus those that attempted multiple flights

Discussion

Our results suggest that honey bee queens neither closely "count" their mates nor modulate their flight behavior based on proximate in-flight energetic costs. These findings seemingly are in contrast to (Schluns et al. 2005) and (Hayworth et al. 2009), respectively. However, there are several details in experimental design that may partially explain these discrepancies. First, the materials of the weights differed among the studies; ferrous magnets were used in (Tarpy and Page Jr 2000) and the current study, whereas none (Schluns et al. 2005) or lead wire (Hayworth et al. 2009) were used in others. It is possible that electromagnetism might explain differences among various studies (see Ferrari 2014), but this possibility remains purely speculative and warrants further empirical testing. Second, the mass and dimensions of our weights were different compared to those used by (Hayworth et al. 2009), as well as the densities of the weights. In addition, Hayworth et al. found that mating number was reduced with a 30 mg weight, but not with a 60 mg weight suggesting that either the sample sizes ($n = 6$) were insufficient or that the effect of increased effort resulting in differential mating is more complex than simply based on effort. Furthermore, the weights used in the current study may possibly have required more effort because they were less streamlined, thus we may not have captured the same range of flight cost tested in the previous study.

In addition to subtle experimental factors, the inefficient proximate process of sperm acquisition by queen bees may also explain the differences in findings. Flight costs appear to be a step function of flight number, not a continuous function based on time or energy expenditure (see Rueppell et al. 2008). It follows, therefore, that weight is not a significant factor in the decision to cease or continue mating behavior, because calculating an optimum does not follow standard cost-benefit models. There is also significant variation in the non-linear relationships among insemination volume, stored sperm, and effective paternity frequency (Delaney et al. 2011; Schluns et al. 2005; Woyke 1962). Therefore, while mating number is a continuous function, the process of acquiring mates is not, and the queens may be assessing how much they have been inseminated rather than counting their mates. Thus it is not surprising that different empirical studies have differed in their findings, with some showing an effect while others

not, because mating number per se is a correlate of the mechanism that queens use to make behavioral decisions of their mating flights.

Based on our current and previous studies, we have developed the following model for the regulation of hyperpolyandry in honey bee queens at the individual, ecological, and evolutionary levels. For the individual behavior of a queen, the total volume of semen with which a queen is inseminated is the major contributor to her subsequent behavior and physiological development. As queens are inseminated on their mating flights, the semen is temporarily stored in the median and lateral oviducts, which swell and likely trigger stretch receptors or similar physical cues. Greater insemination volume affects ovary activation and possibly flight duration as well as her decision to take a second flight. Physical manipulation of the genital track (Koeniger 1981; Nino et al. 2013b) and CO_2 exposure (Mackensen 1947; Nino et al. 2011) also stimulates ovary activation, although the latter is not likely a component in natural mating. These physiological cascades result in differential gene expression in the ovary and brain that alter mating receptivity and reproductive development (Kocher et al. 2008; Kocher et al. 2010), as well as dose-response changes in pheromone profiles (Kocher et al. 2009; Nino et al. 2013a; Richard et al. 2007). Individual variation in the volume, mate number, and physiological threshold leads to the wide, right-tailed variation in mating frequency that is seen among queens (Tarpy et al. 2004).

Ecologically, we contend that queens are not modulating their mating number to immediately address proximate environmental or ecological factors. Even in remote areas, presumably with low drone population densities, queens mate with a sufficient number of males (Tarpy et al. 2015). While local climatic conditions (i.e., weather) obviously can constrain mating, queens do not adjust their mating behavior in response to changing environmental conditions (e.g., newly introduced parasites; (Neumann and Moritz 2000). Instead, queens have likely been selected to achieve a mating frequency above a particular minimum threshold after which the costs are largely inconsequential (Simone-Finstrom et al. 2016).

What seems to be driving hyperpolyandry in our model is selection acting at the evolutionary timescale, and we take a pluralistic view of the adaptive significance of multiple mating. Informed by other honey bee species (Hughes et al. 2008; Oldroyd et al. 1998), we contend that polyandry in *Apis* evolved from monandry because of the genetic load imposed by the sex locus (Page Jr. 1980), then ubiquitous hyperpolyandry evolved to mitigate sweeping disease (Sherman et al. 1988), improved division of labor among the workers (e.g., (Oldroyd and Fewell 2007), and other benefits (reviewed by (Palmer and Oldroyd 2000). As such, differences in levels of polyandry act on an evolutionary level on populations under selection (Franck et al. 2000; Hernandez-Garcia et al. 2009) to modulate the mechanisms that regulate flight behavior (through insemination volume) to ensure mating levels above different threshold mating frequencies adapted to the local environment. However, because mating number is so highly variable and putatively heritable (Kraus et al. 2005), the proximate regulation of mating number per se is not strongly manifested. We believe that this model of the mating system in honey bees provides insights into the significance of a rare but important behavior of social insects.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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