Added Weights Lead to Reduced Flight Behavior and Mating Success in Polyandrous Honey Bee Queens (*Apis mellifera*)

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Abstract:

Honey bee queens are exceptionally promiscuous. Early in life, queens perform one to five nuptial flights, mating with up to 44 drones. Many studies have documented potential benefits of multiple mating. In contrast, potential costs of polyandry and the sensitivity of queens to such costs have largely been ignored because they are difficult to address experimentally. To consider one aspect of mating costs to queens, the difficulty of flight, we compared flight behavior and success among a group of control queens and two experimental groups of queens that carried lead weights of two different sizes. For each queen, we assessed the number and duration of all flights and, after egg-laying commenced, the amount of stored sperm and the number of mates in terms of the offspring's patrilineal genetic diversity. Added weights quantitatively decreased the number of flights, the mean duration of flights and consequently the total time spent flying. Mating success in terms of sperm quantity and patrilines detected among the queens' offspring was also negatively impacted by the experimental manipulation. Thus, it can be concluded that the flight effort of honey bee queens during their mating period is adjusted in response to an experimentally increased cost of flying with multiple consequences for their mating success. Our results suggest that queen behavior is flexible and mating costs deserve more attention to explain the extreme polyandry in honey bees.

Article:

Introduction

The variation in animal mating systems has received a great deal of attention from behaviorists and evolutionary biologists alike (Shuster & Wade 2003). Polyandry, the mating of a single female with multiple males, is evolutionarily derived (Hughes et al. 2008) and relatively rare in social insects (Strassmann 2001). Modest polyandry has evolved in *Vespula* wasps (Ross 1986; Foster & Ratnieks 2001; Goodisman et al. 2002) and several ants, such as *Cataglyphis* (Pearcy et al. 2004), *Cardiocondyla* (Schrempf et al. 2005; Lenoir et al. 2007), *Pachycondyla* (Kellner et al. 2007) and *Plagiolepis* (Trontti et al. 2007). More pronounced polyandry has evolved in *Acromyrmex* and *Atta* leaf cutter ants (Fjerdingstad et al. 1998; Boomsma et al. 1999; Bekkevold et al. 1999; Fjerdingstad & Boomsma 2000; Murakami et al. 2000; Villesen et al. 2002; Sumner et al. 2004), *Pogonomyrmex* harvester ants (Rheindt et al. 2004; Wiernasz et al. 2004) and *Aenictus*, *Dorylus*, *Eciton* and *Neivamyrmex* army ants (Denny et al. 2004; Kronauer et al. 2004, 2007). However, the highest degree of polyandry can be found in *Apis* honey bees (Estoup et al. 1994; Moritz et al. 1995; Palmer & Oldroyd 2000; Wattanachaiyingcharoen et al. 2003; Tarpy et al. 2004). While studies often focus on the maximum number of matings, significant individual variation in mating frequency exists and is rarely explored. For example, mating frequency in the Western honey bee, *Apis mellifera* (L), varies strongly within and between studies (for review see Tarpy & Nielsen 2002), but few studies have sought to systematically investigate this variation (Tarpy & Page 2001; Kraus et al. 2005).

Throughout the genus *Apis*, mating occurs in free flight (Koeniger & Koeniger 1991) and in *A. mellifera* specialized mating sites in the air, known as drone congregation areas (DCAs), are the rule (Winston 1987). DCAs are characterized by a high drone abundance with a highly male-biased sex ratio (Page & Metcalf 1984) and are commonly found at the intersection of preferred drone flight paths without the necessity of conspicuous landmarks, rising above the normal flying height (Loper et al. 1992). Many interconnected DCAs are usually in flying distance of a given colony and both drones and queens fly considerable distances to reach them (Ruttner & Ruttner 1966; Loper et al. 1992). Although a preference for proximal sites in drones has been reported (Koeniger et al. 2005), it is generally assumed that drones and queens fly several kilometers and may visit more than one DCA (Winston 1987). Each DCA is genetically diverse, recruiting from a large number of surrounding hives (Baudry et al. 1998). During mating, drones usually pursue incoming queens in a comet-like formation and the queen mates successively with multiple drones while airborne and then returns to the hive (Gries & Koeniger 1996). However, the details of the queens' natural mating behavior are difficult to observe and thus little is known to date.

Honey bee queens mate after a brief maturation period early in life and store the acquired sperm to fertilize eggs during the remainder of their lives without ever remating. Thus, the quality and quantity of the acquired sperm is of crucial importance for queen fitness by determining colony growth and survival. Queens undertake one to three mating flights (Woyke 1964) and may mate with up to 17 drones on any given mating flight (Adams et al. 1977). Only a small portion of the acquired sperm is transferred into the spermatheca (Winston 1987). Sufficient mating effort should translate into a filled spermatheca with ample sperm for years of offspring production (sperm limitation hypothesis) (Tarpy & Page 2000; Schlüns et al. 2005), and this sperm supply should be of high quality with sufficient genetic diversity. Genetic diversity may improve colony performance either directly (Oldroyd & Fewell 2007) or indirectly (Rueppell et al. 2008). Many studies have found evidence for benefits of multiple mating in terms of the reduction of variance in diploid drone production (Page 1980; Tarpy & Page 2001), disease resistance (Brown & Schmid-Hempel 2003; Seeley & Tarpy 2007) and enhanced division of labor (Oldroyd & Fewell 2007; Mattila & Seeley 2007). These mechanisms are not mutually exclusive and may act synergistically (Rueppell et al. 2008) and there is no doubt that colony genetic diversity and hence multiple mating is beneficial. However, for multiple mating to evolve, these benefits need to outweigh any fitness costs of multiple mating (Koeniger & Koeniger 2007).

In addition to the time and energy expenditure of the mating flights (Tarpy & Page 2000), queens may face considerable fitness costs of mating multiply due to external hazards. The most obvious factors are predation (Kraus et al. 2004; but see Karcher et al. 2008) and the possibility of acquiring sexually transmitted diseases (Yue et al. 2006; de Miranda & Fries 2008). However, other stochastic factors, such as disorientation, accidents or inclement weather are also plausible (Moritz et al. 1995). While a loss of queens during mating flights of 10–20% has been reported (Koeniger & Koeniger 2007), there are few empirical studies directly addressing these mating costs, with the exception of a recent observation of insectivorous birds (Karcher et al. 2008), which suggested such costs to be low.

To evaluate the role of potential costs in the extremely polyandrous mating system of the honey bee, this study was designed to increase flying costs experimentally by gluing weights to queens and to measure the effect on queen flight behavior during the initial mating period and mating success. Experimental weight addition to increase the cost of flying was successfully used to address optimal foraging behavior in honey bees (e.g. Wolf & Schmid-Hempel 1989). It increases the energetic expenditure of flight (per meter and per minute) and may result in less maneuverability. Our results show that added weights significantly decrease queen flights with negative effects on mating success by decreasing the quantity of sperm stored by the queen and the genetic diversity of the offspring. Thus, we conclude that queen flight behavior is flexible and more studies are needed to support the hypothesis that low mating costs permit the high degree of polyandry observed in honey bee queens.

Materials and Methods

Mating Behavior

From May to July of 2007, 120 queens were raised using standard queen rearing procedures (Laidlaw & Page 1997). Two days prior to emergence, individual queen cells were transferred into small queen rearing hives (nucs) containing 400–1000 worker bees. One day before the introduction of the new queen cell, the old queen and any open brood were removed from the nucs. The nucs were checked daily and newly emerged queens were briefly removed from their nuc and randomly assigned to one of the three experimental groups, described below.

Lead wire was custom-cut, weighed on an analytical scale (Mettler AX105, Mettler-Toledo Inc., Columbus, OH, USA), flattened and bent into a U-shape and glued centrally onto the scutum of the queens' thoraxes to avoid interference with wing movements. After a few minutes, to ensure glue hardening, queens were returned to the nucs that they emerged in. Pilot experiments demonstrated that 70 mg of lead weight glued dorsally to the thorax was the maximum amount of added weight with which queens were able to fly. Therefore, we set up two experimental groups (lead weights of 60 ± 3 mg and 30 ± 1.5 mg) and one control group (only glue applied to the thorax), with 40 queens in each group. Although all queens were well-attended by workers and no aggression towards queens was observed during regular hive inspections, 20 queens of the 60 mg group, 19 of the 30 mg group and 1 of the control group were either lost or without their weights before the onset of data collection, and were thus excluded from the analysis.

The experimental nucs were distributed randomly throughout our apiary with a minimum inter-hive distance of 5 m. To observe queen flight activity, a wooden runway (12 cm × 12 cm) with a plexiglass cover was attached to each hive to serve as the only entrance/exit. This runway could be blocked with a queen excluder by placing it in one of two slots, arranged proximal and distal to the hive entrance/exit. While the queen was in the hive, the queen excluder was kept in the distal position. When the queen was observed in the runway, trying to leave her nuc, she was permitted to exit by moving the queen excluder to the proximal slot. The queen excluder was then kept in the proximal position until the queen was found in the runway, returning from her flight. She was then permitted to enter the nuc by transferring the queen excluder again to the distal slot.

After preliminary observations had suggested that queens in our apiary flew between 15:00 h and 17:00 h, the entrances were monitored every 5 min, daily between 14:30 h and 18:30 h for the entire experimental period. The departure and return times of each queen were observed for approximately 20 d after emergence to determine the overall number of flights for each queen, the duration of flights and total time spent on orientation and mating flights (which were not distinguished further). Some returning queens landed and moved underneath the hive or into the screened ventilation hole and consequently were noticed by the observer only much later. The corresponding flights had an unknown return time and were recorded as censored flight observations with a minimum duration of 5 min (Laidlaw & Page 1997).

After a queen initiated flights, her nuc was observed daily for production of worker offspring. Once a sufficient number of offspring were produced, 20 offspring were randomly removed from the colony along with the queen. These samples were immediately frozen and stored at −20°C for later analysis of the sperm content of the sperm storage organ (spermatheca) and the offspring genetic diversity.

Quantification of Sperm

From the control and both experimental groups, we randomly selected six queens among those that had gone on mating flights and produced female offspring. The abdomens of these queens were dissected to remove the spermatheca. Heads and thoraxes remained frozen for later DNA extraction. The spermatheca was placed in 50 µl of Kiev buffer on a microscope slide. On the slide, forceps were used to rip open the spermatheca and allow the sperm to mix with the buffer solution. The dissolved sperm were transferred by pipette into a 1.5 ml centrifuge tube. The slide and spermatheca were rinsed four more times with additional Kiev buffer (total volume 450 µl) that was collected in the same 1.5 ml tube to ensure that all of the sperm were collected. The contents of each tube were mixed vigorously by vortexing for a minimum of 5 min to reduce sperm clumping.

To estimate the total number of sperm in the spermatheca, all sperm cells in 0.8 µl of this solution were counted. We used a standard hemocytometer (two independent 1 mm^2 blocks from each of four replicate samples drawn out of the well-mixed solution) and counted only the *a priori* determined end of the thread-like sperm cells. The resulting number was multiplied by 625 to account for the total volume of the solution.

Genotyping and Estimation of Mating Frequencies

DNA was extracted from the same 18 queens used for the sperm quantification (six from each group) and 20 offspring per queen. Using a standard Chelex® (BioRad, Hercules, CA, USA) extraction protocol, we suspended a small, lateral slice of each larva and one leg of each queen in 100 µl of 5% Chelex® solution. Fourteen unlinked microsatellite loci (Solignac et al. 2007) were screened for amplification and the level of allelic diversity in 18 larvae (one from each colony). Five loci (SV204, SV257, Ac001, K0357B (Solignac et al. 2007) and one new microsatellite on scaffold 5.10 named OR5_10: forward primer: 5′-TCGTGCAATGAGATCTTTCG-3′, reverse primer: 5′-CGACTCAACAATGTCAGCTTG-3′) were selected for analysis. The overall allelic diversity of the chosen microsatellite loci ranged between 4 and 13 alleles (Table 1). Thus, the markers provided sufficient detection power to compare mating frequencies, although our absolute numbers may be slight underestimates. All 18 queens and their offspring were genotyped at these five loci with a tailed-primer approach (Schuelke 2000), using IRD-labeling for detection on LiCor's 4200 DNA Analyzer (Lincoln, Nebraska). Alleles were amplified with a touchdown PCR protocol, decreasing the annealing temperature from 68°C to 48°C (Schug et al. 2004). PCR reactions were carried out in 10 µl and contained 1 ng of template DNA, 200 µm dNTPs, 0.25 µm forward primer, 0.5 µm reverse primer, 50 nm of IRD-labeled M13 primer, 2 mm $MgCl_2$, 1× PCR buffer and 0.2µ of Taq polymerase. PCR products of different size and label were combined and analyzed on 25 cm gels with 1000 V for 2–3 h. Genotypes were scored in duplicate. The minimum number of mates was determined manually for each queen by excluding queen alleles and counting unique paternal multi-locus allele combinations among the offspring. As males are haploid, each mating results in one unique haplotype, when excluding the maternal contribution to the offspring.

Table 1. Microsatellites used to study offspring genetic diversity

Locus	Allelic diversity (no. of alleles)	Size (bp)	Chromosome
K0357B	11	174–214	3
SV204	9	196–224	4
OR5_10	13	230–254	5
Ac001	9	204–226	7
SV257	4	150–156	13

Results

Overall, queens went on 1.4 ± 1.4 (SD) flights on average with a range of 0–6. The experimental treatment had a significant effect [one-way anova $F_{(2,77)} = 4.2$, $p = 0.018$]: the weight addition quantitatively decreased the number of queen flights (Fig. 1). Dunnett's T3 *post-hoc* tests revealed that the 60 mg group went on significantly fewer flights than the control group ($p = 0.005$), with the 30 mg group intermediate but not significantly different from the control group ($p = 0.606$) or the 60 mg group ($p = 0.135$).

The average duration of queen flights was 43.0 (95% C.I.: 29.4–56.6) min, estimated from 77 uncensored (71%) and 32 censored flight records. Overall, 31 queens (12 of 39, 7 of 21 and 12 of 20 queens in the 0, 30 and 60 mg treatment groups, respectively) were not observed flying and excluded from subsequent analyses. Increased weight significantly shortened the average duration of flights (Mantel-Cox Log Rank test: $\chi^2 = 6.5$, $df = 2$, $p = 0.040$; Fig. 2a). The average flight in the control group lasted 56.7 (33.8–79.6) min, in the 30 mg group 35.9 (19.1–52.6) min and in the 60 mg group 22.9 (10.3–35.5) min. Consequently, the product of the number of flights and average flight duration, the total time spent flying, also significantly declined ($\chi^2 = 10.8$, $df = 2$, $p = 0.004$; Fig. 2b) from the control group [131.2 (90.7–171.8) min] to the 60 mg group [42.4 (13.1–71.7) min], with the 30 mg group intermediate [81.3 (46.7–115.9) min].

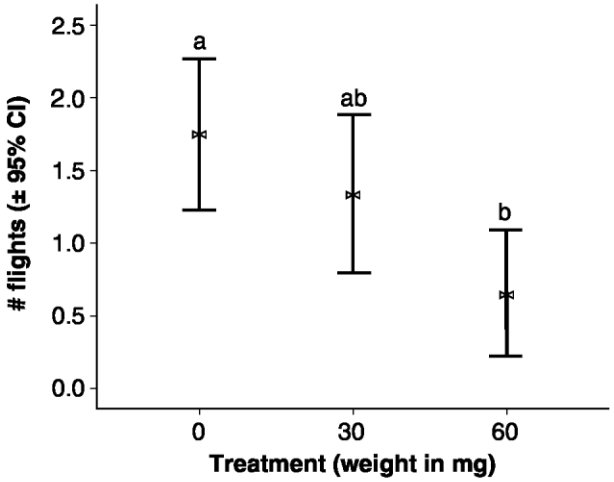

Fig. 1: Means and 95% confidence intervals of the number of all flights taken before egg-laying initiation for one control (0 mg) and two experimental groups (30 and 60 mg) of *Apis mellifera* queens carrying different sizes of lead weights (0 mg: N = 39; 30 mg: N = 21; 60 mg: N = 20). Lowercase letters refer to groups that were significantly different as determined by Dunnett's T3 *post-hoc* test.

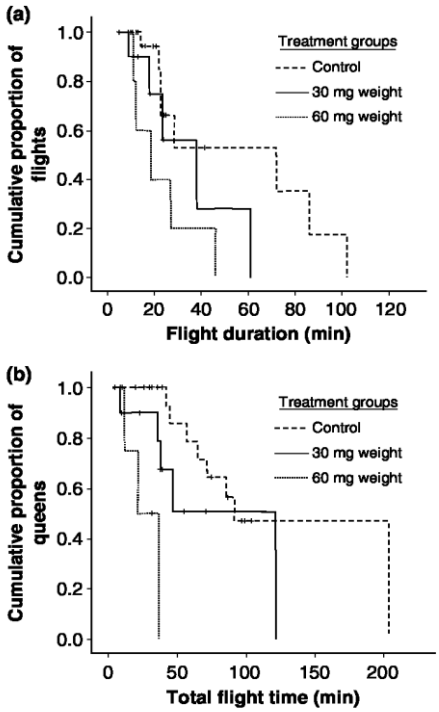

Fig. 2: The duration of individual flights (a) and the overall time spent flying by queens (b) displayed as cumulative survival graphs. (0 mg: N = 27; 30 mg: N = 14; 60 mg: N = 8). Thirty-one queens were never observed flying and were consequently excluded from this analysis. Crosses represent censored data due to a missing record of start or end time of a flight.

Mating success of the queens in terms of the amount of sperm the queens stored in their spermatheca was not significantly different among treatment groups [one-way anova: $F_{(2,15)} = 1.3$, $p = 0.311$; Fig. 3]. In contrast, mating success in terms of the genetic diversity of the offspring was significantly different among treatment groups. The experimental weights significantly decreased the patrilineal genetic diversity of the offspring produced in terms of patrilines represented [$F_{(2,15)} = 5.7$, $p = 0.014$] and paternal allelic diversity [$F_{(2,15)} = 10.3$, $p = 0.002$; Fig. 4], although Dunnett's T3 *post-hoc* tests indicated that only the differences between the control and the 30 mg treatment groups were statistically significant ($p = 0.004$) but not between control and 60 mg group ($p = 0.166$) or between the two experimental groups ($p = 0.124$). The average number of detected patrilines was 9.3 (8.0–10.6) in the control group, 6.3 (5.4–7.0) in the 30 mg group and 7.8 (6.3–9.4) in the 60 mg group. Across all groups, the number of detected matings was not significantly correlated with the amount of sperm in the spermatheca (Spearman's $R = 0.19$, $n = 18$, $p = 0.440$).

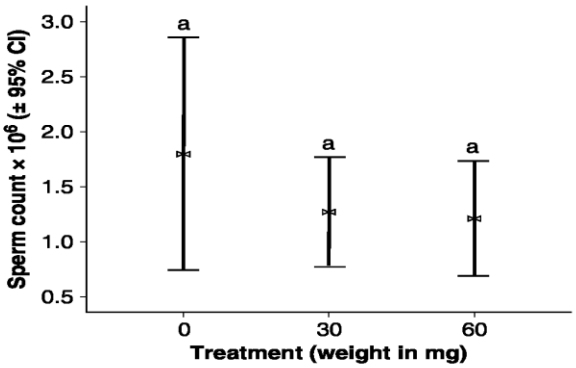

Fig. 3: Means and 95% confidence intervals of the number of sperm stored in the queens' spermatheca when egg-laying commenced, comparing *Apis mellifera* queens among control (0 mg) and two weight-bearing (30 and 60 mg) treatment groups (n = 6 in each group). Lowercase letters refer to groups that were significantly different as determined by Dunnett's T3 *post-hoc* test.

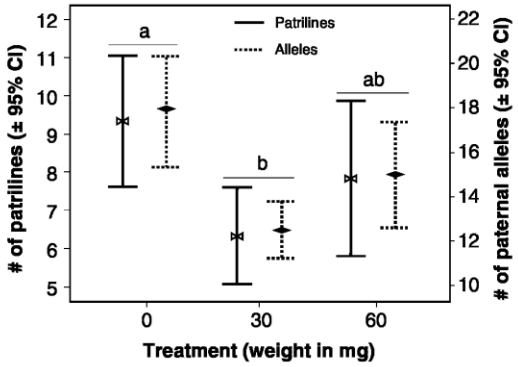

Fig. 4: Means and 95% confidence intervals of the number of patrilines represented in genotyped offspring (20 offspring per queen) and the number of paternal alleles across all loci (five microsatellites) comparing *Apis mellifera* queens among control (0 mg) and two weight-bearing (30 and 60 mg) treatment groups (n = 6 in each group). Lowercase letters refer to groups that were significantly different as determined by Dunnett's T3 *post-hoc* test.

We assessed the effects of treatment and flight time on the amount of sperm and the number of patrilines with two independent ancovas with treatment as main, fixed effect and flight time a covariate. The first analysis indicated a treatment effect on the amount of sperm (control > 30 mg > 60 mg) when flight time was statistically controlled for [$F_{(2,15)} = 4.1$, $p = 0.048$]. Flight time itself showed a significantly negative relation to the amount of stored sperm [$F_{(1,15)} = 15.4$, $B = -4.9 \pm 1.2$, $p = 0.002$]. Flight time was not significantly correlated to the number of mates [$F_{(1,15)} = 0.6$, $p = 0.458$] and therefore dropped from the model which thus contained only the already reported effect of treatment (see above). We also evaluated the correlation between sperm quantity and the number of patrilines, which was non-significant ($R = 0.137$, $n = 18$, $p = 0.589$).

Discussion

Animal mating patterns evolve in response to fitness costs and benefits (Shuster & Wade 2003). The exceptional polyandry of several social insect lineages has prompted numerous studies on potential benefits of multiple mating for social insect queens (Page 1980; Mattila & Seeley 2007; Oldroyd & Fewell 2007; Seeley & Tarpy 2007; Rueppell et al. 2008). Our study is the first to experimentally address the cost of mating, demonstrating in the honey bee model that queen flight behavior is responsive to increased flying costs. The resulting shorter and fewer flights affected together with direct effects of the experimental manipulation the mating success of queens in terms of stored sperm quantity and the genetic offspring diversity.

Behavioral flexibility of honey bee queen mating behavior has been studied before to evaluate the sperm limitation hypothesis for multiple mating. Results were mixed with one study reporting that queens seek additional mating opportunities when their spermatheca is not filled in accordance with the hypothesis (Schlüns et al. 2005) and another study rejecting this scenario (Tarpy & Page 2000). A third study found a negative correlation between flight time and sperm content of the queens, supporting the hypothesis that queens assess their mating status and adjust their mating behavior accordingly (Koeniger & Koeniger 2007). The same negative relation between flight time and sperm content is found in our study and our findings further support the notion that queens are behaviorally flexible and adjust the number and length of their flights based on perceived costs and benefits of mating. The shortening of flights may be explained as a direct consequence of the increased energetic demand for weighted queens, forcing queens to prematurely terminate mating flights. However, the decision for or against additional flights is made by queens and workers (Hammann 1957) in the hive free of energetic constraints, subject only to the evaluation of relative costs and gains. Our results suggest that this evaluation takes place.

Both, stored sperm quantity and the offspring's genetic diversity are negatively impacted by the weight addition. However, the effects are different and sperm quantity and the number of detected patrilines show no significant correlation. Together with other studies (Tarpy & Page 2000, 2001; Schlüns et al. 2005; Koeniger & Koeniger 2007), our results raise the possibility that queens control sperm transfer to their spermatheca and deliberately limit the amount of retained sperm per copulation when mating conditions are good but allow more sperm to enter their spermatheca when conditions are bad and mating is perceived to be costly. This new hypothesis remains to be tested but it could resolve some of the disagreement about the sperm limitation hypothesis and potentially explain the negative correlation between the amount of stored sperm and flight time in this and other studies (Koeniger & Koeniger 2007).

Our sperm estimates are below estimates from earlier studies (Woyke 1964) but in accordance with other studies (Schlüns et al. 2005). The discrepancies between studies may be due to technical errors, but they could also reflect true biological differences (Koeniger & Koeniger 2007). Our calculations provide minimum estimates because we neglected that the spermatheca itself adds a small volume to the total volume in which the sperm was diluted but our conclusions remain valid because our treatment groups were compared relative to each other. Conversely, our mating flight estimates were longer than that of other studies (Ruttner 1954; Koeniger & Koeniger 2007). It is possible that the extreme records of flight duration may actually represent two consecutive flights. Due to our observation schedule, we cannot exclude the possibility that a queen returned briefly (<5 min) to the entrance platform of her hive before leaving on a second mating flight. However, this was never observed and there is no convincing argument against queen mating flights to exceed 30 min. In fact,

the observation that queens fly more economically than drones (Gmeinbauer & Crailsheim 1993) and drone flights of up to 160 min have been recorded (cited in Gmeinbauer & Crailsheim 1993) argues against such a theoretical limit to the extent that queens and drones are comparable. In contrast to other studies, our study was set up in an urban area with low colony density which may have forced longer mating flights (Koeniger & Koeniger 2007). Finally, we have to acknowledge the difficulty of finding a valid experimental manipulation to alter mating costs. Added weight may not be ideal but it is a non-invasive, simple and quantifiable procedure (Wolf & Schmid-Hempel 1989) that is presumably relatively insensitive to environmental conditions. It entails an energetic cost and decreases the queens' flight capability and thus increases mortality risks such as predation or death from exhaustion or disorientation, similar to the effect of naturally poor flight conditions, including strong winds. However, added weights might also change the in-hive behavior of queens and their treatment by workers. Therefore, we cannot exclude indirect worker effects as a potential explanation for the observed results but this potential involvement of workers would not negate our main conclusions.

Our results support the view that honey bee queen mating behavior is cost sensitive (Koeniger & Koeniger 2007). Queens and/or their workers are capable of adjusting the mating behavior in response to the trade-off between the costs and benefits of multiple mating. Individual differences in assessing this trade-off may contribute to the pronounced variation in honey bee queen mating frequency (Moritz et al. 1995; 1996). Although queen mating flights may be costly (Schlüns et al. 2005; Koeniger & Koeniger 2007), the associated costs and risks differ among queens and may consequently be perceived differently (Fjerdingstad & Keller 2004). Individual differences may explain why queens in the 60 mg treatment group did not differ significantly in mating number from the other two groups despite significantly shorter flight times. Alternatively, this could be explained by an altered queen flight pattern in this group that decreased drone search time. In general, the mating costs for honey bee queens seem to be sufficiently low to be outweighed by the benefits of multiple mating. In other species, relatively high mating costs may help the maintenance of single mating (Rueppell et al. 2008).

Some costs, such as the risk of disorientation, level off after the first successful mating flight and the number of matings per flight may be relatively cost-neutral. Extremely high mating frequencies therefore may not require a special explanation (Schlüns et al. 2005). However, our experimentally increased costs of mating resulted in a significant reduction of the degree of polyandry in honey bees, emphasizing that mating costs are an important variable for the evolution of mating systems. Thus, mating systems that minimize costs of polyandry, such as the honey bee drone congregation areas, could play an important role in facilitating the evolution of extreme polyandry in some social insects but further studies are needed to evaluate this hypothesis.

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