



Multiple nuptial flights, sperm transfer and the evolution of extreme polyandry in honeybee queens

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The honeybee, *Apis mellifera*, has an extremely polyandrous mating system, which often involves multiple nuptial flights by its queens. To understand the evolution of extreme polyandry, we investigated the cost of multiple nuptial flights in relation to potential benefits. We analysed, with eight DNA microsatellite loci, the paternity of worker offspring of naturally mated queens. Queens that were restricted to one nuptial flight, but may have taken additional nuptial flights if allowed to do so, had significantly fewer matings than queens that started oviposition after a single nuptial flight. Furthermore, the number of sperm stored in a spermatheca increased significantly with the number of matings. We suggest that queens adjust their nuptial flight frequencies according to their mating success in their previous nuptial flights. The number of copulations seems to serve as a signal for the initiation of oviposition. In the light of these findings, we reconsider and discuss the significance of the sperm limitation hypothesis for the evolution of extreme polyandry in *A. mellifera*.

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The evolution of polyandry in the social Hymenoptera has raised considerable interest since multiple mating distorts the potential benefits of a close intranidal relatedness predicted by inclusive fitness theory (Crozier & Pamilo 1996). Furthermore, multiple mating may be costly for the individual queen if there is a mating risk (Moritz & Southwick 1992). More than a dozen hypotheses have been presented to explain the evolution of polyandry despite these potential costs (e.g. Keller & Reeve 1994; Oldroyd et al. 1998; Cole & Wiernasz 1999; Jennions & Petrie 2000; Crozier & Fjerdingstad 2001; Tarpay & Page 2001). The genetic variance hypotheses have been regarded as highly plausible (Crozier & Page 1985; Palmer & Oldroyd 2000; Crozier & Fjerdingstad 2001; Tarpay & Page

2001). According to these hypotheses, genotypically more variable colonies have enhanced fitness correlates (e.g. more effective division of labour or increased disease resistance). However, Cole (1983), putting forward the sperm limitation hypothesis, argued that, because degree of polyandry and colony size are positively related in several species, ant queens heading populous and long-lived colonies need large semen stores to maintain the colony size. The sperm limitation hypothesis has been empirically supported in leafcutter ants. In *Atta sexdens*, the spermatozoa in young adult males are equivalent to only about 13–30% of the number of sperm in the spermathecae (=female sperm storage organs) of newly mated queens (Kerr 1962). Furthermore, Fjerdingstad & Boomsma (1998) reported that multiple mating increases the sperm stores of *A. colombica* leafcutter ant queens. In this species queens are long lived (10–16 years), and their colonies can consist of more than 2 million individuals (Fjerdingstad & Boomsma 1998).

In the Western honeybee, *Apis mellifera*, queens mate in flight in drone congregation areas in a rapid sequence consecutively with several drones (Koeniger et al. 1979). The nuptial flights are performed about a week after adult emergence and the queens do not mate again after the onset of oviposition (cf. Winston 1987). Even though on

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nuptial flights queens run a considerable risk of not returning to the colony (15–30% of flights on average; Ruttner 1980), they mate multiple times and perform up to five mating flights (Roberts 1944; Alber et al. 1955).

If multiple mating is adaptive and has been shaped through natural selection, we would predict that honeybee queens should optimize their behaviour by minimizing the number of nuptial flights while maximizing the number of matings. Depending on the mating success of previous nuptial flights, the queen should 'decide' whether to conduct further nuptial flights or initiate oviposition instead. However, Tarpy & Page (2000) reported a complete lack of behavioural control of the queens over their mating frequency. They found no difference in the degree of polyandry between queens that deliberately started oviposition after the first nuptial flight and queens that were caught before their second nuptial flight. In the latter, oviposition was initiated with carbon dioxide gas and the mating frequency was derived from the genotypes of the worker offspring. This is an elegant approach because one does not experimentally interfere with the natural copulation process.

Obtaining sperm is an ultimate reason for female mating. In honeybees, the sperm limitation hypothesis has widely been rejected (Crozier & Page 1985) because a single drone has in principal enough sperm to fill a queen's spermatheca completely (Moritz & Southwick 1992). However, when a researcher instrumentally inseminates a honeybee queen with semen of a single drone, only up to 20% of the spermatheca is filled (Woyke 1960). Indeed, Woyke (1960) reported an increase in sperm numbers in a spermatheca when injecting higher semen volumes into the queens' oviducts during instrumental insemination. However, inseminating queens with more than 8 µl of semen yielded essentially no further increase in stored sperm, probably because of the limited storage capacity of the spermatheca (Woyke 1960). The function describing the dependency of the sperm number in the spermatheca on the amount of injected semen must therefore fulfil two basic requirements. (1) There must be an upper limit set by the carrying capacity of a spermatheca and (2) the graph of the function must pass through the origin because injection of no semen clearly results in no spermatozoa in a spermatheca. The linear or logarithmic regressions (as used by Woyke 1960, 1988) do not fulfil these requirements. The most parsimonious equation with only two parameters matching the above requirements is as follows:

$$S(V) = \frac{a}{b+V} - \frac{a}{b} \quad (1)$$

where $S(V)$ = sperm number in the spermatheca, V = semen volume injected, a , b = regression constants and $-a/b$ = capacity limit.

In the case of no insemination, this function has the intrinsic property of resulting in zero sperm and for large amounts of semen the function converges to the maximum sperm number that a honeybee queen's spermatheca can hold. Reanalysing Woyke's (1960) data, we found that equation (1) yields a highly significant regression relationship ($N = 10$, $R^2 = 0.97$, $F_{2,8} = 1353.3$,

$P < 0.001$). If there is a similar effect under natural mating conditions, it is very suggestive that a queen requires a certain minimum number of matings to fill her spermatheca completely and to ensure a lifetime supply of sperm needed to maintain a large colony.

In this study, we measured the number of copulations and the number of nuptial flights of queens (as in Tarpy & Page's 2000 study), and also evaluated the number of spermatozoa transferred to the spermatheca after the queen has started oviposition. First, we tested the hypothesis that queen honeybees can control their mating frequency. We compared 'control' queens, which started oviposition after only a single nuptial flight, with 'test' queens, which were constrained to a single flight but tried to perform additional flights. We also analysed the number of matings in control queens with various numbers of nuptial flights to test whether the number of matings increases with more flights. Finally, we tested the sperm limitation hypothesis by studying the impact of the mating frequency of a queen on the number of sperm stored in her spermatheca.

METHODS

Multiple Nuptial Flights and Mating Frequency

Experimental design

Twenty-one virgin honeybee sister queens, *A. m. carnica*, reared at the same time in the same breeder colony were placed in small colonies ('mating nucleus' of ca. 2000 workers) at the Technical University near the city centre of Berlin, Germany. The experiment was set up in the middle of the local honeybee mating season (June 1997). The flight entrances of the hives were equipped with Perspex-covered runways (15 × 15 cm) and a queen excluder to prevent unobserved flights of queens (workers can pass through this screen; Koeniger 1976). Two observers patrolled the flight entrances continuously during the daily mating period (1200–1700 hours). Whenever a queen appeared in the runway, the queen excluder could be removed to allow for a flight. After a queen returned from her flight the excluder screen was reinstalled to prevent unobserved flights. We examined each queen for the 'mating sign' (secretions of male accessory glands in her sting chamber) as an indication of a copulation when she returned to the hive.

The 21 experimental queens were divided into a test and a control group after the first nuptial flight of all queens. We randomly chose five test queens when they were trying to leave their hive for a second nuptial flight. These five test queens were anaesthetized with CO₂ to induce oviposition (Mackensen 1947). Subsequently they were reintroduced into their colonies, where they started egg laying. All other queens were unconstrained (control group) and allowed to perform any number of nuptial flights. After the last nuptial flight, we observed both control and test colonies on a daily basis for 14 days.

After the queens had produced sealed brood, we genotyped the worker brood to determine the mating frequency.

The queens were then killed with CO₂ to determine the number of spermatozoa in their spermathecae. Their spermathecae were excised, the semen diluted, and the spermatozoa counted in a Fuchs-Rosenthal counting chamber using phase-contrast microscopy (Ruttner 1976). Ten replicate counts were made per spermatheca, and we used the arithmetic mean as an estimate of the number of stored spermatozoa.

Genotyping and estimates of mating frequencies

All queens and a sample of 35 worker offspring per queen were genotyped with eight DNA microsatellite loci (A7, A8, A24, A28, A35, A88, A113, B124) using standard PCR protocols (Solignac et al. 2003). DNA primers were labelled with fluorescence dyes for detection in an automated DNA sequencer (ABI Prism 310 Genetic Analyzer, Applied Biosystems, Applied Biosystems, Darmstadt, Germany) using the chemicals and the protocols of the supplier.

Using Mendelian rules, we derived the genotypes of the siring drones from the genotypes of the worker offspring and of the queens. Since all queens were sisters we also used this pedigree information. If both alleles of a worker at a given locus were identical with those of the mother queen, the exact genotype of the father could not be identified. In these cases we conservatively assigned paternity using the information from the other loci in a way that minimized the number of patrines observed in the colony. The number of patrines or fathers, respectively, that sire the worker offspring equals the number of observed matings. To correct for not detecting a drone because of the finite sample size of the worker offspring analysed per queen (nonsampling error), we computed the estimated number of matings according to Cornuet & Aries (1980).

$$o = k - k \left(1 - \frac{1}{k}\right)^n$$

where n is the sample size of the worker offspring, o is the number of observed matings, and k is the number of estimated matings.

The effective number of matings, P_e , based on the frequencies of the patrines and corrected for the sample size, was computed according to Pamilo (1993):

$$P_e = \frac{(n-1)}{\left(n \sum_{i=1}^l q_i\right) - 1}$$

where n is the sample size, l is the number of siring drones, and q_i is the proportion of workers sired by the i th drone. The effective number of matings of a queen, which is usually lower than the observed number of matings, is the mating number if all drones are represented equally within her offspring.

Statistical analyses

A sire drone may by chance share an identical genotype with another siring drone. The probability of not detecting a patrine because of identical sire genotypes

(nondetection error, P_r), and thus underestimating the number of matings, was calculated according to Boomsma & Ratnieks (1996). We applied Student's t tests to compare mating frequencies of the different groups of queens with a Bonferroni adjustment to the level of significance using STATISTICA (StatSoft 2001).

Mating Frequency and Sperm Number

Experimental design

We investigated the influence of the number of queen matings (independent variable, irrespective of the number of flights) on the sperm number stored in the spermathecae (dependent variable). The CO₂ treatment of the test queens had no effect on sperm transfer from the lateral oviducts into the spermathecae, because it was done at least a day after mating took place (Woyke 1988). This allowed us to combine the data from both test and control queens. In addition, we included another set of queens ($N = 12$) that were mated on the North Sea island of Baltrum (Neumann et al. 1999a). As described above, we determined mating frequencies (Neumann et al. 1999a) and counted the sperm.

Statistical analyses

We used equation (1) to estimate the impact of the mating frequency on the sperm number stored in the spermathecae. We modified this equation by replacing the semen volume $S(V)$, with the estimated mating frequency $S(\text{estimated matings})$ in the nonlinear regression procedure of the STATISTICA software package, applying the classic Gauss–Newton algorithm for least squares.

RESULTS

Multiple Nuptial Flights and Mating Frequency

Number of nuptial flights

All 21 queens performed their nuptial flights in the same narrow time window (11–13 June 1997) at the same location in Berlin, Germany. Three queens did not return from their nuptial flights, showing a local mating risk of about 14%. Eight of 13 queens of the control group deliberately started oviposition after a single nuptial flight; the other five performed multiple nuptial flights (Table 1).

The five test queens were caught at the flight entrances when they tried to perform a second nuptial flight. They started egg laying within a week of the CO₂ treatment and produced worker offspring with the exception of one queen (test queen 5; Table 1). Although this queen had a mating sign after her nuptial flight that unambiguously indicates at least one copulation, she had no sperm in her spermatheca.

Mating frequency

Owing to the large number of variable microsatellite loci tested, the nondetection error was less than 0.0004.

Table 1. Number of nuptial flights, number of sperm in the spermatheca and mating frequencies of the experimental queens

	No. of flights	Spermatozoa ($\times 10^6$)	Observed matings	Estimated matings	Effective matings
Control queens (single flight)					
1	1	1.38	10	10.3	7.08
2	1	1.50	11	11.5	8.62
3	1	2.74	11	11.5	12.04
4	1	1.73	12	12.7	6.26
5	1	0.47	14	15.5	10.26
6	1	2.32	15	17.1	14.51
7	1	2.16	16	18.8	16.08
8	1	2.71	16	18.8	19.83
Mean \pm SE		1.88 \pm 0.27	13.13 \pm 0.85	14.53 \pm 1.22	11.88 \pm 1.67
Control queens (multiple flights)					
9	2	1.03	8	8.1	7.44
10	2	0.95	12	12.7	10.26
11	2	3.11	14	15.5	9.60
12	3	3.55	11	11.5	10.44
13	3	1.60	18	22.7	24.79
Mean \pm SE		2.05 \pm 0.54	12.6 \pm 1.66	14.1 \pm 2.46	12.50 \pm 3.12
Test queens					
1	1	0.22	6	6	1.89
2	1	1.75	9	9.2	4.84
3	1	1.16	10	10.3	8.04
4	1	1.97	11	11.5	6.69
5	1	0	$\geq 1^*$	$\geq 1^*$	0
Mean \pm SE		1.02 \pm 0.40	7.40 \pm 1.81	7.60 \pm 1.89	4.29 \pm 1.49

Control queens: $N = 13$; unforced single or multiple flights. Test queens: $N = 5$; restricted to one nuptial flight despite their attempts to perform a second flight, and oviposition initiated with CO_2 treatment.

*This queen had a mating sign upon return, and thus at least one copulation.

Within the control group there was no significant difference in the number of estimated matings between the queens that started oviposition without enforcement after a single nuptial flight ($N = 8$) and those control queens performing multiple nuptial flights ($N = 5$, Student's t test: $t_{11} = 0.173$, $P = 0.866$; Table 1).

To analyse the impact of the mating frequency on the number of nuptial flights, we compared the control queens, which started oviposition after only a single nuptial flight ($N = 8$), with the test queens, which were constrained to a single flight but tried to perform additional flights. We included only those test queens that started laying fertilized eggs after the CO_2 treatment ($N = 4$) and omitted the test queen that had no sperm in spite of a copulation. The number of estimated matings was significantly lower in the restricted test queens which were prevented from performing another nuptial flight (estimated matings: $t_{10} = 2.72$, $P = 0.022$; Bonferroni adjusted level of significance: $P < 0.025$; Table 1).

Mating Frequency and Sperm Number

All queens that had conducted at least one nuptial flight (confirmed through either the presence of a mating sign or the production of worker offspring) were included in the analysis ($N = 30$). Table 1 and Fig. 1 show the mating frequency estimates and the sperm number counts of the

queens' spermathecae. The nonlinear regression analysis (see equation 1) of sperm number in a spermatheca on the degree of polyandry yielded a highly significant fit ($N = 30$, $R^2 = 0.373$, $F_{2,28} = 81.7$, $P < 0.001$; Fig. 1). The regression equation was as follows: $S = (-13.704) / ((5.07915) + [\text{estimated matings}]) + 2.968$. The regression coefficients ($a = -13.704 \pm 14.5$; $b = 5.07915 \pm 4.1$) estimate a capacity limit ($-a/b$) of 2.968 ± 5.03 million

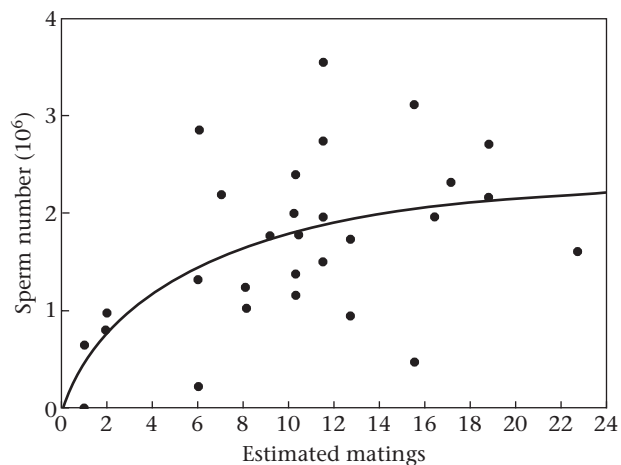


Figure 1. Regression curve of the number of sperm in the spermatheca (dependent variable) on the number of estimated matings (independent variable) for all 30 queens.

spermatozoa per spermatheca. Omitting the two queens that had a mating sign but no semen in the spermathecae (one in our first data set and one in the second set from Neumann et al. 1999a), neither change the significant regression ($N = 28$, $R^2 = 0.206$, $F_{2,26} = 78.6$, $P < 0.001$) nor significantly affected the estimate of the carrying capacity of the spermatheca ($-a/b = 2.49 \pm 5.06$).

DISCUSSION

Our data show that queens that were restricted to a single nuptial flight mated with significantly fewer drones than queens with an unrestricted number of nuptial flights. This supports the hypothesis that queens have behavioural control over their mating frequencies. Furthermore, queens that mated more frequently had more spermatozoa in their spermathecae. Thus, queens have to mate with many males to ensure a complete filling of their spermathecae, indicating that the sperm limitation hypothesis is a plausible cause for the evolution of extreme polyandry in honeybees.

Multiple Nuptial Flights and Mating Frequency

Mating frequency estimates

Both sample size and allele frequencies in the population determine the precision of the mating frequency estimate (Boomsma & Ratnieks 1996). In our study, the error of not detecting a patriline in the sample was less than 0.0004. Thus, the resolution of the eight DNA microsatellite markers is adequate. The number of patrilines not sampled in a finite sample (nonsampling error; Cornuet & Aries 1980) underlies the assumption that all drones contribute equally. Although this may not be true in honeybees (Moritz 1986), all three measures of mating frequencies applied in this study (Table 1) reveal similar results, showing that our sample sizes yielded robust estimates for the degrees of polyandry. Although the effective number of males and the resulting intracolony relatedness may have an impact on selection at the colony level (see genetic variance hypotheses above), the effective number of males is unlikely to be under queen control. Hence, in this study the number of copulations of the queen seems to be most important, because that is what the queen can potentially measure or control besides the number of nuptial flights.

Behavioural control over mating frequency

The number of nuptial flights alone is irrelevant for the initiation of oviposition (Peer 1957; Koeniger 1976). Furthermore, the filling of the spermatheca can be excluded as the only cue because queens do not initiate oviposition after instrumental insemination without CO₂ treatment (Mackensen 1947). Koeniger (1981) was able to initiate oviposition of queens that had matings manipulated to prevent semen transfer, showing that the copulation itself is an important factor for oviposition. All of our test queens had copulated but nevertheless tried to

perform additional nuptial flights. However, they had fewer matings than control queens, although they had similar numbers of spermatozoa (Table 1). Therefore, our results corroborate Koeniger's (1981) notion that copulations, and not the filling of a spermatheca, play the key role in triggering the egg-laying phase.

At first glance our results appear to contradict those of Tarpy & Page (2000), who performed a similar study in the Sacramento Valley in California, U.S.A. (coincidentally in the same year). Tarpy & Page (2000) did not detect a statistically significant difference in the number of matings between constrained and unconstrained queens. Nevertheless, the queens hindered on further nuptial flights in their study had fewer (although not statistically significant) effective matings. Tarpy & Page (2000) observed rather few copulations per queen in comparison to numerous other studies (e.g. Estoup et al. 1994; Kryger & Moritz 1997; Neumann & Moritz 2000; Palmer & Oldroyd 2000; this study). These low estimates in combination with high variances may have resulted in the lack of a statistically significant result. In principle, it seems difficult to conclude from a small, but nonsignificant difference that there is no difference. Fitness differences may be small, but still evolutionarily relevant. Furthermore, in Tarpy & Page's (2000) experiment only eight of 30 queens (27%) tried to perform more than one nuptial flight, whereas we had 10 of 18 queens (56%) in Berlin doing so (2×2 table; $\chi^2_1 = 4.01$, $P = 0.045$). Thus, the conditions of the mating experiments or the animal stocks might have been different in the two studies, leading to diverging results.

Mating Frequency and Sperm Number

Honeybee copulations are densely packed in time and space, taking place in localized drone congregation areas where many drones mate with a queen in quick succession (Koeniger et al. 1979). Therefore, we fully agree with Tarpy & Page (2000) that natural selection is much more likely to operate on the number of nuptial flights (which have a high risk) than on the number of individual copulations (with a low risk). We suggest that the queen may have a threshold for a certain number of matings 'to evaluate' her mating success. This threshold is likely to be subject to the local environmental conditions, such as drone density and weather (Alber et al. 1955; Neumann et al. 1999a, b; Kraus et al. 2004). As soon as she has reached the threshold, she will not perform any further nuptial flights and will initiate oviposition. Clearly, this threshold can be dramatically exceeded on any given nuptial flight because the queen may mate at a low cost with many drones on her last nuptial flight. Such a mechanism would plausibly explain both the high variance of polyandry observed in naturally mated *A. mellifera* queens (range 1–45; Moritz et al. 1996; Neumann et al. 1999a) and the extreme degree of polyandry observed in the genus *Apis* (Palmer & Oldroyd 2000).

Two of 30 queens in our study showed a mating sign but had no sperm in their spermathecae. Since the mating sign is a clear indication of a copulation, the risk of mating

with a drone that is not transferring any sperm was very high. Therefore, multiple mating in honeybees should also be considered as a safeguard mechanism, because queens are probably not able to assess the quality of their drone partners before mating.

Our results on sperm numbers and matings in naturally mated queens closely resemble those of Woyke (1960) on instrumentally inseminated ones. Both studies indicate that a certain threshold of matings (inseminations) is required to fill the spermatheca to its storage capacity. Indeed, although individual drones produce sufficient sperm to fill a spermatheca, more than 97% of the drone's semen is expelled after insemination (Koeniger & Koeniger 2000). Even given that evolution drives the amount of semen per drone to a maximum, a trade-off with other fitness-related morphological and physiological male traits (e.g. body size, Schlüns et al. 2003; flight navigation, speed and endurance) seems highly probable.

Queens obtaining insufficient semen suffer dramatic fitness disadvantages because they start laying drones and the colony soon dies (Moritz & Southwick 1992). One objection against the sperm limitation hypothesis is that queens eject most of the semen they receive during mating (Simmons 2001). However, if morphological constraints in the female genital organs result in inefficient semen transfer from the lateral oviducts to the spermatheca (Kraus et al. 2004), we find it difficult to exclude this hypothesis as a plausible explanation for the evolution of extreme polyandry at this stage. Multiple mating might be an evolutionary response to the inefficient semen transfer mechanism, providing insurance against low-quality males.

The need for large sperm stores to maintain populous and long-lived colonies can be generally predicted in the social insects. Indeed, there is a tendency for an increase in spermatheca volume with increasing level of sociality in bees (Apoidea; Martins & Serrão 2002). Furthermore, there is a positive correlation between sperm numbers of colony-founding queens and total cell numbers of mature nests in yellowjackets (*Vespa* and *Dolichovespula*; Stein & Fell 1994). Further study is needed of the queen's semen handling in species that establish populous and long-lived colonies but in which the queens are only singly mated, such as in many stingless bees (Meliponinae; Palmer et al. 2002).

At this point, we certainly cannot and do not want to reject the genetic variance hypotheses predicting general fitness benefits from polyandry at the colonial level. These models have received convincing support in the harvester ant *Pogonomyrmex occidentalis* (Cole & Wiernasz 1999). However, it appears notoriously difficult in honeybees to detect clear-cut fitness advantages in groups of workers that are highly genotypically variable (Neumann & Moritz 2000; Palmer & Oldroyd 2000). Recently, intranest thermoregulation has been shown to be positively correlated with intragroup genetic variance (Jones et al. 2004). However, Jones et al. (2004) acknowledged that polyandry probably evolved in honeybees for reasons other than the task allocation system. In general, all genetic variance hypotheses do well in explaining up to six matings but they fail to explain more than 10 matings, which are frequently observed in honeybees (Palmer & Oldroyd 2000). In the

light of the dramatic fitness consequences of insufficient fecundity in poorly mated queens and the weak effects of genotypic variability on colony fitness, we think it is premature to discard the sperm limitation hypothesis for the evolution of extreme polyandry in honeybees.

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