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ORIGINAL ARTICLE

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Sperm utilization pattern in the honeybee (*Apis mellifera*)

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Abstract Queen honeybees (*Apis mellifera*) mate with a large number of drones on their nuptial flights. Not all drones contribute equally to the queen's offspring and the queen's utilization pattern of spermatozoa from different drones has an important impact on the genetic composition of the colony. Here we study the consequences of sperm use for the fitness of the queen's mates with microsatellite DNA-fingerprinting. Eight queens were instrumentally inseminated with semen of six or seven drones. Each drone contributed either 0.5 µl or 1.0 µl semen, respectively, and we analyzed both the impact of the insemination sequence and the amount of semen on the sperm utilization. Our data show no significant effect of the insemination sequence but a strong impact of the semen volume of a drone on the frequency of his worker offspring in the colony. This effect was not linear and the patriline frequencies of the drones contributing larger semen volumes are disproportionately enhanced. If these observations are also valid for natural matings, drone honeybees should maximize the number of sperm but not apply specific mating tactics to be first or last male in a mating sequence.

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Introduction

Multiple matings by females have received much attention in behavioral ecology (Arnqvist and Nilsson 2000; Knight 2002) since they have great consequences pertaining to sperm competition and sexual selection (Andersson 1994; Simmons 2001). Sperm competition, the competition between sperm from two or more males for the fertilization over a given set of ova (Parker 1970a, 1998), is a widespread phenomenon in arthropods and vertebrates (Møller and Birkhead 1998; Simmons 2001). By multiple mating and setting the stage for sperm competition, females may increase the probability that their eggs are fertilized by competitively superior sperm (Simmons 2001). Sperm competition can also result in morphological, physiological or behavioral adaptations in males, e.g. mating plugs or mate guarding. Thus, many male adaptations are related to the avoidance of sperm competition. Last male precedence (i.e. the disproportional use of the sperm of the last mating male) is a common mode of sperm competition in insects (Parker 1970b; Simmons 2001). A potential mechanism is the displacement of sperm by the second male to mate (Parker 1970b). Sperm competition can also lead to the increase of testes weights and higher sperm production (Harcourt et al. 1981; Møller 1988; Harcourt 1997).

Multiple mating, a prerequisite for sperm competition, is intensively studied in social insects because of its impact on genetic relationships among nest mates, which is of great importance concerning the inclusive fitness theory (Hamilton 1964; Ratnieks et al. 2001). The honeybee (*Apis*) exhibits the most extreme degree of polyandry (Koeniger and Koeniger 2000; Palmer and Oldroyd 2000) among the few genera in social insects with a regularly polyandrous mating system (Strassmann 2001). Mating frequencies of up to 44 matings per queen have been reported for *Apis mellifera* (Moritz et al. 1996).

Honeybees mate in flight at so-called drone congregation areas. More than 10,000 drones can be present at a drone congregation area (N. Koeniger, unpublished data). The operational sex ratio is strongly male-biased since honeybee colonies produce thousands of drones and only few virgin queens during the mating period (Winston 1987). Many drones fly in a "comet" behind the queen to get the best position for mating (Gries and Koeniger 1996). In sharp contrast to the queens, drones are strictly monogamous because they inevitably die during the copulation (Koeniger et al. 1979). Drones inject their sperm into the queen's lateral oviducts (Winston 1987), where they are stored before they reach the spermatheca via the *ductus spermaticus*. Sperm transfer in honeybees is mediated by the semen pump (Bresslau 1905) and by active spermatozoal movements (Ruttner and Koeniger 1971). The stored sperm can survive within the spermatheca for many years until the queen dies. Cryptic female choice in the broad sense ("nonrandom paternity biases resulting from female morphology, physiology, or behavior that occur after coupling" Pitnick and Brown 2000) cannot be excluded in the honeybee but it seems to be rather unlikely that queens can preferentially select sperm from specific drones once the semen pool is stored in the lateral oviducts. Nevertheless, postcopulatory selection on ejaculates can occur in honeybees in case of incompatibility at the sex locus. Drones having an identical sex allele to one of the two alleles of the queen have a 50% reduced chance of producing female offspring (Mackensen 1951).

Polyandry in social insects is mostly considered as being advantageous either directly to the queen, or to the colony as a whole (Crozier and Fjerdingstad 2001; Tarpy and Page 2001). The latter includes the queen, her daughters (the workers, and offspring queens) and her sons (the drones). However, the paternal perspective, i.e. the fitness consequences of multiple mating for the queen's mates, are rarely taken into consideration (Baer 2003). Yet, there could be extreme post-mating selection among the siring drones. Although there is no indication of a sperm incapacitation process in the oviducts (Woyciechowski and Król 1996), sperm of different drones stored in the spermatheca may not be used at equal frequencies for egg fertilization by the queen (Page 1986). Considering the reproduction of the drones, both individual and colony selection can come into play. On the one hand, the drone fathers of the colony could benefit from potentially increased fitness of the polyandrous queen and her colony; however, they could encounter strong intrasexual competition. Indeed, the male mating success of honeybee colonies can vary over more than an order of magnitude, even given that the same numbers of drones are present in the colonies (Kraus et al. 2003). Surprisingly, there is some evidence that the mating sign a drone leaves in the queen's sting chamber rather promotes copulation than hinders it (Koeniger 1990).

Most morphological, physiological or behavioral traits of drones are readily interpreted as being optimized for mating and reproducing. For instance, in several Asian honeybee species (Radloff et al. 2003) drone flight prowess (as measured by the excess power index, Hepburn et al. 1998) is superior to that of workers. *A. mellifera* drones also have larger eyes, and the numbers of olfactory poreplate sensilla on the antennae are much higher in drones than in the female castes (see Winston 1987 and references therein). Traits designed for direct fighting with other drones are missing and, indeed, direct contests among drones during mating have not been reported. This lack does, however, not exclude potential postcopulatory selection among drones. Because the queen mates multiple times and stores ejaculates from many males, the stage for sperm competition is set. One possible mechanism for postcopulatory competition is variation in sperm numbers. Such natural variation is documented in the honeybee. Differences in sperm numbers are reported among drones of European and African origin (Rinderer et al. 1985). There is also substantial variation in sperm production among two drone morphs (small and large drones, Schlüns et al. 2003) that can appear at the same drone congregation areas (Berg 1991). These differences in sperm numbers provide variation for selection to operate on after insemination has occurred.

Sperm utilization in honeybees has been repeatedly studied but in no case could the early claims of sperm clumping by Taber (1955) be repeated (Page and Metcalf 1982; Laidlaw and Page 1984; Moritz 1986; Page 1986; Haberl and Tautz 1998). Franck et al. (1999, 2002) reported on a decrease of the variance of subfamily frequencies (=patriline frequencies) over time, probably as a result of increased sperm mixing in the spermatheca.

The impact of the mating sequence has been addressed in few studies in honeybees (Laidlaw and Page 1984; Moritz 1986) but these studies had used inbred mutant lines, which might have different male fitness and therefore mask the potential effect of the insemination sequence. Studies that did use neutral markers (Franck et al. 1999) did not control for semen volume, which again may have masked a potential last-male advantage. We therefore controlled semen volumes, along with precise assessment of patriline frequencies, in order to determine the queen's utilization of sperm from different drones. Thus both potential differences arising from varying drones' fertilities due to differences in ejaculate volume (as found under natural conditions) and the potential effect of the drone's position in the insemination sequence are taken into account.

Methods

Carnolian honeybee queens (n=8; *A. m. carnica*) were instrumentally inseminated with 4.0 µl semen each (Moritz 1989). This volume is about a third of the average semen volume that is found in the queen's oviducts after a nuptial flight (Woyke 1960). The Carnolian drones used for the insemination of each respective queen were unrelated to each other. In order to be able to precisely measure the semen volume, a calibrated 5.0-µl glass capillary was attached to the insemination syringe. One queen was sequentially inseminated by seven drones, one contributing 1.0 µl semen (double volume) and the others 0.5 µl (single volume). All other queens were inseminated with semen of six drones; four drones contributing 0.5 µl (single volume) and two drones contributing 1.0 µl (double volume). Thus, all “single volume” drones contributed 12.5% of the total volume and all “double volume” drones contributed 25%. The position of those drones contributing the double volume in the insemination sequence was randomized.

Worker brood samples were taken from every queen 32 days after insemination. DNA was extracted according to Walsh et al. (1991) using one leg per pupa. All drones used in the inseminations (*n*=49) were genotyped at eight microsatellite loci (A107; A113; A14; A35; A88; A28; B124; A76; Solignac et al. 2003) using standard PCR protocols (Estoup et al. 1994, 1995). DNA primers were labelled with fluorescence dyes for detection in an automated DNA sequencer using the protocols of the supplier (ABI Prism 310 Genetic Analyzer). If workers were heterozygous and paternity could not be assigned because the queen was also heterozygous having the same alleles (i.e. two drones had the same two alleles by chance as the queen), workers were again genotyped at further microsatellite loci until paternity could be unequivocally determined. Sperm utilization was studied in respect of the amount of semen contributed by a specific drone and in respect of his position in the sequence of insemination. Chi-square analyses and multiple linear regression analyses were performed to test the influence of both the drone’s position and the semen volume on the sperm utilization. In order to analyse the effect of the semen volumes of the drones on their number of offspring, the data are pooled among the queens. Thus, potentially deviating sperm utilizations of individual queens are not taken into consideration. All statistical tests were done with the STATISTICA software (Statsoft 2001).

Results

In total, 745 honeybee-worker pupae were genotyped. From every queen, on average 93 daughter workers were analysed (range 87–96). All workers could be unequivocally assigned to one of the potential drone fathers. The patriline frequencies, defined as the proportion of the worker offspring of a particular drone relative to the total number of workers analysed of one specific queen, are given in Table 1.

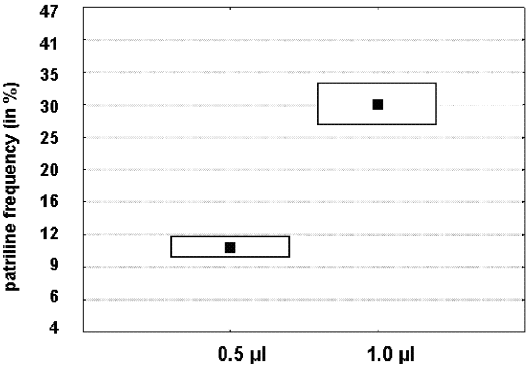

Fig. 1 Means and standard errors of the patriline frequencies of the single-volume patrilines (0.5 µl; *n*=34) and the double-volume patrilines (1.0 µl; *n*=15)

The frequency distribution of the “single volume” patrilines (0.5 µl; *n*=34) deviates significantly from a normal distribution (Lilliefors *P*<0.01). Therefore, all data were angular transformed, which is especially appropriate to percentages (Sokal and Rohlf 1995). The multiple linear regression analysis (Table 2) allows a comparison of the relative contribution of both insemination sequence and semen volume to the prediction of the patriline frequency. Only semen volume has a significant influence on the patriline frequency (Fig. 1). About 54% of the variance of the patriline frequencies are explained by the semen volume of the respective drone. There is, however, no significant impact of the drone’s position on the patriline frequency (Fig. 2; *P*=0.08).

The mean frequency of workers sired by drones (*n*=34) that contributed 12.5% of the semen injected into a queen is 11.16±1.0%. In contrast, the mean frequency of workers sired by drones (n=15) that contributed 25.0% of the semen injected into a queen was 28.03±2.8%. A chi^2-test

Table 1 Percentages of patrilines and total numbers of workers genotyped for each queen. The numbers of the drones refer to the sequence in the instrumental insemination procedure. Thus, drone 1 was the one whose semen first entered the queen’s reproductive tract. Numbers marked with *asterisks* indicate that double semen volume of the particular drone was used for insemination

	Queen 1	Queen 2	Queen 3	Queen 4	Queen 5	Queen 6	Queen 7	Queen 8
Drone 1	25.0	15.2	8.4	8.3	6.9	43.2*	27.4*	30.3*
Drone 2	5.2	10.9	10.5	8.3	51.7*	23.2*	6.3	11.2
Drone 3	18.8	22.8*	14.7	10.4	6.9	4.2	9.5	21.3
Drone 4	25.0*	4.3	22.1	39.6*	16.1*	20.0	34.7*	5.6
Drone 5	11.5	17.4	28.4*	24.0	6.9	4.2	10.5	23.6*
Drone 6	7.3	29.3*	15.8*	9.4*	11.5	5.3	11.6	7.9
Drone 7	7.3							
Total	96	92	95	96	87	95	95	89

Table 2 Multiple regression analysis: depending variable: angular transformed proportions of patrilines; independent variables: drone’s position in insemination sequence and sperm volume injected (single: 0.5 µl; double: 1.0 µl) (*n*=49; *R*=0.733; R^2=0.537; *F*(2,46)=26.663; *P*<0.001)

	β	SE of *β*	*B*	SE of *B*	*t*(46)	*P*
Constant			0.161723	0.051674	3.12967	<0.01
Position	−0.178845	0.100344	−0.014492	0.008131	−1.78231	0.0813
Sperm volume	0.712490	0.100344	0.441274	0.062147	7.10046	<0.001

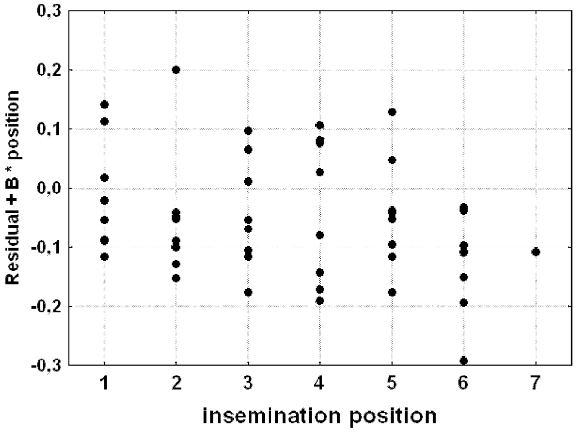

Fig. 2 Partial residuals of the insemination position effect (n=49 patrilines)

revealed that the patrilines fathered by the drones contributing a double volume of semen are significantly over-represented compared to patrilines descending from drones contributing a single dose [single volume (0.5 µl): observed 355, expected 396.5; double volume (1.0 µl): observed 390, expected 348.5; n=745; χ^2=9.26; P<0.01; df=1].

Discussion

Our data unequivocally show uneven and non-random sperm utilization by queens inseminated with sperm of several drones. Patriline frequencies strongly depend on the semen volume of the respective drone, which may not be that surprising. Sperm competition operating just by the number of sperm has also been demonstrated in *Drosophila melanogaster* (Pitnick et al. 2001), and more recently in the cricket *Gryllus bimaculatus* (Gage and Morrow 2003). Sperm utilization in honeybees is, however, not just random sampling of the injected semen, because of the significant over-representation of drones contributing larger semen volumes. These drones sired 28% of the offspring in contrast to the expected 25%. This seems to be a minor difference but even small differences may be evolutionarily significant in the long run. Cryptic female sperm choice of specific males serving as a potential cause of differential sperm usage is not possible in our experimental set-up because queens were anaesthetized and did not encounter the drones.

The lack of a significant effect of the insemination order on the patriline frequencies in our data is consistent with previous reports with mutant drones and queens (Laidlaw and Page 1984; but see Moritz 1986). If there was any effect at all, which was not detectable in our data, the magnitude of the effect was clearly much smaller as compared to the impact of semen volume. Since the mating sign does not serve as a mating plug in honeybees and there is no contest competition among drones to achieve copulations, we suggest that the rank in the insemination sequence may be largely selectively neutral. Franck et al. (2002) came to the same conclusion when comparing patriline frequencies in the colony and the genotype of the last mating drone. Clearly all interpretations of the results are based on the assumption that the artificial insemination using a syringe leads to a comparable sperm utilization pattern as would be achieved under natural mating conditions.

We conclude from our study that evolution forces drones to produce a maximum number of sperm. Even if sperm contribution had merely a linear impact on paternity, there would be positive selection for increased sperm numbers. The disproportionate paternities of drones contributing more semen, however, amplifies the effect. Positive selection for increased sperm numbers could be driven by sperm competition, which is almost inevitable since the sperm of many drones have a very high temporal overlap within the spermatheca. The approximately equally sized bumble bee males (*Bombus terrestris*) that lack sperm competition (because of the monandrous queen) produce only about 0.5 million spermatozoa, which is more than an order of magnitude less than sperm numbers in *A. mellifera* drone bees (Moritz 1981; Baer and Schmid-Hempel 2000; Schlüns et al. 2003).

An alternative reason for higher sperm numbers in honeybees than in bumble bees may be the need for more sperm. In honeybees, the sperm-transfer mechanism is inefficient. Only 3–5% of the sperm of an individual drone reach the spermatheca (Koeniger and Koeniger 2000). Thus, the spermatheca will not be completely filled if the honeybee queen is inseminated with ejaculates of too few drones (Woyke 1960), and hence the queen has to mate repeatedly (Kraus et al. 2004). It is well established that queens can run out of sperm within their life time, causing the worker bees to replace her with a new queen (Ribbands 1953; Winston 1987). An alternative explanation to the sperm-limitation hypothesis has been put forward, interpreting the queen's semen pump served as a sperm-mixing tool (Laidlaw and Page 1984; Page 1986). Both hypotheses are, however, not mutually exclusive. Queen honeybees have a longer life time than bumble-bee queens and produce enormous numbers of eggs. The spermatozoa in their spermathecae are kept alive for years. Thus, selection pressures among males may be confounded with the need for long-living sperm. Indeed, a comparative study by Hunter and Birkhead (2002) revealed a higher viability of honeybee sperm compared to bumble bee sperm. Viable spermatozoa are adaptive to drones with or without male-male competition for the fertilization of ova. The longer sperm live, the longer a colony can be maintained and the greater the chance for a drone to sire a new gyne. However, both reasons—viability selection and sperm competition—are not mutually exclusive. High selective pressures due to sperm competition and viability selection may have driven drone honeybees in the evolutionary past to produce many spermatozoa of high viability.

The large amount of semen produced by the drones could be traded-off by other traits that also play important roles in mating and reproduction (e.g. flight ability of drones). This could prevent drones from investing even more in sperm production.

Aside from large sperm numbers, sperm competition could also favor large sperm sizes (if competitive benefits of sperm size increase with increasing number of sperm in competition, Parker 1993). This correlation was reported to apply to three bumble bee species (Baer et al. 2003). The phylogenetically closely related honeybee (sperm length 313 μm, Woyke 1983) fits in well with sperm lengths being much longer than those found in *B. terrestris* (about 170 μm, Baer et al. 2003), and they are still considerably longer than sperm lengths in the moderate polyandrous *B. hypnorum* (2–4 matings, sperm length 210 μm, Baer et al. 2003).

There have been intense and long discussions on the conundrum of polyandrous queens and monogynous drones in honeybees (Palmer and Oldroyd 2000; Tarpy and Page 2001). About a dozen hypotheses have been put forward as ultimate causes for this peculiar mating system (Crozier and Fjerdingstad 2001). The most difficult problem with the honeybee's mating system is the extraordinarily high level of polyandry. Queens often copulate with substantially more than ten drones (Estoup et al. 1994; Kryger and Moritz 1997; Neumann and Moritz 2000) and also the genetically effective number of matings often exceeds ten (Tarpy and Nielsen 2002; Kraus et al. 2004). The enhancement of intracolonial genotypic variability is especially often considered as a plausible reason for multiple mating (e.g. Palmer and Oldroyd 2003; Tarpy 2003). Genetic variance models lose, however, sufficient explanatory power if queens mate with more than six drones (Palmer and Oldroyd 2000). Queens showing an equal usage of the sperm of all drones, and hence maximizing the effective paternity, should be favored by natural selection. Thus, our results do not support the genetic variance hypotheses, because we find unequal sperm usage and disproportionate frequencies decreasing the effective number of matings.

The dependency of paternities of workers on semen volume may not necessarily hold for paternities of new queens reared by the colony for reproduction. Different patriline frequencies in workers and gynes have been reported (Tilley and Oldroyd 1997). The ultimate fitness advantage of drones would imply that they were well represented in the newly reared queens in the colony. Since we tested no queens, we cannot exclude that nursing bees preferentially do not foster the queen offspring of the more spermatozoa-producing drones. With our experimental set-up, we could not test for this aspect, since we manipulated the amount of semen experimentally from randomly taken drones. To address this specific issue in more detail, further research must employ natural variation in sperm numbers, as is found between drones of European and African origin (Rinderer et al. 1985) or within populations between large and small drones (Schlüns et al. 2003).

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