Male fitness of honeybee colonies (*Apis mellifera* L.)

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Abstract

Honeybees (*Apis mellifera* L.) have an extreme polyandrous mating system. Worker offspring of 19 naturally mated queens was genotyped with DNA microsatellites, to estimate male reproductive success of 16 drone producing colonies. This allowed for estimating the male mating success on both the colony level and the level of individual drones. The experiment was conducted in a closed population on an isolated island to exclude interferences of drones from unknown colonies. Although all colonies had produced similar numbers of drones, differences among the colonies in male mating success exceeded one order of magnitude. These differences were enhanced by the siring success of individual drones within the offspring of mated queens. The siring success of individual drones was correlated with the mating frequency at the colony level. Thus more successful colonies not only produced drones with a higher chance of mating, but also with a significantly higher proportion of offspring sired than drones from less successful colonies. Although the life cycle of honeybee colonies is very female centred, the male reproductive success appears to be a major driver of natural selection in honeybees.

Introduction

Measuring fitness in a population of social insects is a difficult task, because selection operates at least at three distinct levels: (1) direct individual selection (classical Darwinian selection), (2) indirect individual selection (through kin selection, Hamilton, 1964), and (3) directly at the colony level (Moritz & Fuchs, 1999). Colonies typically are hermaphrodites producing both male and female reproductives (Moritz & Southwick, 1992) commonly with a strong numeric bias for either sex (Crozier & Pamilo, 1996). The reproductive success at the colony level depends on both the number of sexual reproductives produced, and the individual reproductive success of each queen or male. In social insect species with colony fissioning, e.g. honeybees (genus *Apis*), stingless bees (tribe Meliponini) and army ants (genus *Eciton*), the female reproductive success is determined through the number of surviving reproductive colonies (swarms) produced and can therefore be relatively easily measured (Wilson, 1971; Moritz & Southwick, 1992). The determination of the reproductive success of the males is much more difficult, since males are usually short lived and matings are difficult to observe (Wilson, 1971). This task becomes even more complex in species with polyandrous mating systems, like in wasps (genus *Vespula)*, myrmicine ants (genus *Atta*) or honeybees (genus *Apis*, Boomsma & Ratnieks, 1996).

The mating system of the western honeybee (*Apis mellifera*) is characterized by large drone congregation areas, where males from many colonies assemble to mate with virgin queens which visit the congregation areas on their nuptial flights (Ruttner, 1988; Gary, 1992). Whereas male honeybees (drones) can only mate with a single queen and die after mating, *A. mellifera* queens can mate with up to 45 drones (Moritz *et al.*, 1995, 1996; Neumann & Moritz, 2000). The male reproductive success of a whole colony is not determined by the plain number of males produced in a colony (up to 5000 drones per colony and mating season, Winston, 1987). Drones of one colony may out-compete others in mating efficiency (Berg *et al.*, 1997). The picture is further

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complicated because the queens store a semen supply of many drones in their spermatheca (Palmer & Oldroyd, 2000) and the contribution of individual drones to this semen pool is not equal (Moritz, 1986), resulting in considerable differences in patriline frequencies. Offspring queens are more likely reared from frequent patrilines which results in fitness differences among the siring drones. Thus the individual male reproductive success and the reproductive success of his mother colony, both depend on the intracolonial patriline frequencies in the offspring colony.

Only a few studies have dealt with male fitness and reproductive success of social insect colonies in natural populations (Berg *et al.*, 1997; Sundström & Ratnieks, 1998; Sundström & Boomsma, 2000). Although it is possible to determine the reproductive success of individual males, it is difficult to assess the male reproductive success of a whole colony. This can only be determined if the queen genotypes of the colonies contributing to the male gene pool are known. Although highly variable DNA microsatellites have been developed for paternity analyses in honeybees (Estoup *et al.*, 1993, 1994, 1995) it is virtually impossible to evaluate the reproductive success at colony level for honeybees under natural conditions, because the number of colonies contributing can be very high (Ruttner & Ruttner, 1972; Baudry *et al.* 1998). In this study we therefore used a controlled experimental set-up on an isolated North Sea island, which was free of other honeybee colonies. We controlled for the number of drone producing colonies, the number of drones per colony and the number of virgin queens available for mating. This closed population approach allowed for a precise assessment of the male reproductive success on the individual as well as on the colony level.

Materials and methods

Honeybee management and sampling

Drone colonies

During two mating seasons 16 naturally mated sister queens ($r = 0.25$) of *A. mellifera* (eight queens in 1999 and eight in 2000) were introduced into strong, equally sized colonies (drone colonies) on the island of Neuwerk. The island was otherwise completely free of any honeybee colonies and more than 8 km away from the mainland ensuring its reproductive isolation (Klatt, 1929; Neumann *et al.*, 1999). Each drone-producing colony was composed of 20 frames covered with workers and ten frames of worker brood. All foreign drones were removed from the colony by using a queen excluder (Dadant, 1992). Each colony received two empty drone cell frames, which were readily used by the queens for producing drone eggs, and allowed for ample drone production. In all drone colonies, the 16 queens produced approximately an equal number (4000–5000) of sexually mature drones during the mating period. To infer the genotypes of the drone producing queens, sealed drone brood was sampled from each of the 16 drone colonies and stored in ethanol at −20 °C until DNA processing.

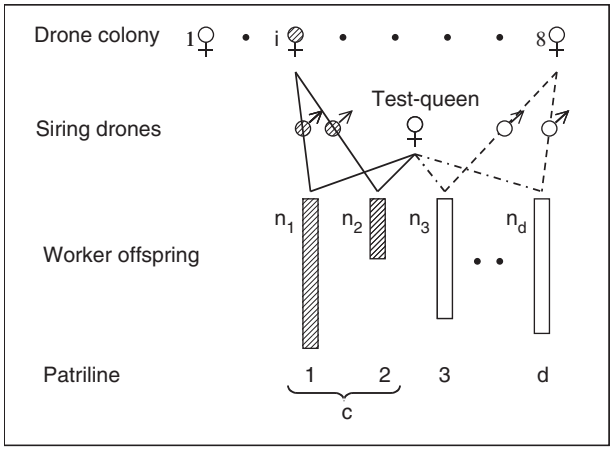

Fig. 1 Pedigree of drone colonies with test queens. n = number of offspring per patriline. c = number of patrilines (matings) of drone colony i present in a given test – queen, d = total number of patrilines (matings) in a given test – queen.

Virgin queen colonies

Additional to the drone colonies, 19 small 'nuclei' colonies (ca. 2000 workers, no drones) with virgin sister queens (test queens, $r = 0.25$, unrelated to the drone providing queens), were placed on the island in early June 1999 (six nuclei colonies) and June 2000 (13 nuclei colonies). All test queens were of the same age and conducted unrestricted mating flights during the same 4-week period. To quantify the male reproductive success of the drone colonies, the test queens' offspring and the test queens themselves were genotyped (for pedigree see Fig. 1). For this purpose the test queens and sealed worker brood samples were taken from each test queen colony. The brood samples and the test queens were stored in ethanol at −20 °C until DNA processing.

DNA analysis and estimation of parameters

Genotyping

DNA was either extracted from pupal flight muscle tissue of 20–30 individuals per colony or from the flight muscle tissue of the adult test queens, using routine protocols (Walsh *et al.*, 1991). The individuals were genotyped with nine DNA microsatellite loci (A7, A24, A35, A43, A76, A88, A107, A113, B124 in 1999 and, A7, A24, A28, A79, A88, A107, A113, A35 and IM in 2000) developed by Estoup *et al.* (1993, 1994, 1995) and Rowe *et al.* (1997). DNA was amplified with fluorescent dye labelled primers (FAM, HEX and TET) in the PCR and allelic DNA

fragment sizes were scored in two electrophoresis runs comprising either loci A7, A35, A43, A76 and A113 (set 1) or A24, A88, A107 and B124 (set 2) in 1999 and A7, A24, A35, A88 (set 3) or A28, A79, A107, A113, IM (set 4). The electrophoresis was carried out in an automated DNA sequencer (ABI 310) using Genotyper© software and the protocols of the supplier with a TAMRA labelled internal size standard (GeneScan 500).

Identification of genotypes and patrilines

The allele combinations of the drone producing queens were inferred from the genotypes of their male brood. By genotyping 20 male pupae per queen, the chance of missing an allele of a queen at a given locus (in case of heterozygosity) was less than $P = 0.001$ and the genotype of the drone producing queens could be identified with certainty. The test queen genotypes were identified by genotyping the queens themselves.

By genotyping the worker offspring (20–30 pupae) of the test queens, the number of patrilines within a test queen offspring was determined and the relative frequency of a given patriline calculated. The chance of missing a patriline because two drones by chance had the same genotype (nondetection error) was calculated based on the genotypes of the drone producing queens (similar to Boomsma & Ratnieks, 1996). The number of patrilines in a given sample of worker offspring equals the number of observed matings $d_o$due to finite sample sizes the number of observed matings d_o may underestimate the actual number of matings (nonsampling error). Therefore we calculated the estimated number of patrilines (estimated matings) following Cornuet & Aries (1980):

$$d_o = d_e - \left[d_e \left(1 - \frac{1}{d_e} \right)^n \right] \quad (1)$$

where d_o = number of observed matings, d_e = number of estimated matings, n = sample size.

With a given worker sample size n and a given number of observed matings d_o it is thus possible to approximate the number of estimated matings d_e via iteration. If the differences between the number of estimated matings d_e and the number of observed matings d_o is small, the majority of patrilines in a given sample of worker offspring has been detected.

As every patriline in a queens offspring represents one drone she mated with, the mother colonies of the successfully mated drones could be inferred by comparing the patriline allele combination with the genotypes of the drone producing queens (Fig. 1). Identical allele combinations of a patriline with a possible drone producing queen, identified this queen as the mother of that particular patriline.

Estimating male mating frequency at the colony level

The male mating frequency of a colony depends on the number of successfully mating drones of that colony (a single drone can only mate once). In some cases it was not possible to assign a drone genotype to a single drone colony, and more then one colony could be the potential source of a siring drone. In these cases each potential mother colony received an equal share of this mating. For example if three drone colonies qualified as the potential origin of a given patriline, each of these colonies received 0.33 matings. The total male mating frequency of a drone colony was determined by adding all its matings with all test queens.

Estimating the reproductive success of individual drones

Different drones do not contribute equally to the offspring of a given queen. As a drone can only mate once, his reproductive success depends on the frequency of his offspring in the colony (patriline frequency). The higher the frequency of a given patriline is, the higher are the chances of having a new queen reared from this particular patriline. The relative siring success, s, of an individual drone was therefore calculated from the deviation of the number of worker offspring he sired from the expected number under equal distribution of patrilines within a given test queen's offspring:

$$s_k = \frac{o_k}{n} - \frac{1}{d_o} \quad (2)$$

where s_k = relative siring success of drone k, o_k = numnumber of offspring sired by drone k, d_o = observed number of matings of a test queen, n = number of workers sampled.

For example if a drone sired ten out of a total sample of 20 workers and the queen mated with four drones, the siring success of this drone was 25% higher than expected under equal distribution resulting in a relative siring success of $s = 0.25$.

All statistical analyses were performed using the *Statistica*© software package.

Results

All drone mother genotypes could be unambiguously identified. A total of 147 patrilines (observed matings d_o) were identified in the sample of 482 genotyped workers. All patrilines could be assigned to the drone colonies present on the island. A total of 113 patrilines could be unambiguously assigned to a single drone colony. Between two and four drone colonies could be identified as potential drone sources for the remaining 34 patrilines. The probability for not detecting a patriline because the siring drone by chance had the same allele combination as another one from the same drone colony (nondetection error) was at the average 0.026 ± 0.019 depending on the heterozygosity of the drone mother (Table 1). This resulted on the average in only 4.3 undetected patrilines in the entire sample of 147 patrilines. The number of estimated matings,

Table 1 Genotypes of the drone producing queens at the microsatellite loci used for the analysis. All microsatellite alleles are given in the length of their fragments (in bp). Also shown is the probability that a drone by chance had the same genotype as another one from the same colony (nondetection error, nde).

	Microsatellite locus									
Drone mother	A7	A107	A113	A24	A88	A35	A43	A76	B124	nde%
1999										
D1	101/112	158/172	211	97	147	109	135/137	280/288	214/227	0.031
D2	101/112	168/172	211/217	97	147	109	121/135	278/288	212/214	0.016
D3	112	158/172	211	97	147	109	121/135	280/288	212/214	0.063
D4	112	164/168	211	89/97	138/147	109/111	135/137	268/280	218/227	0.008
D5	99/112	164/168	211	97	138/147	109	135/137	268/280	212/227	0.016
D6	101/112	164/168	211	97	147	109	135	268/280	212/217	0.063
D7	112	168/172	211	89/97	138/147	109/111	135/137	268/280	212/218	0.008
D8	99/112	164/168	211	97	138/147	109	135/137	256/280	212	0.031
2000	A7	A107	A113	A24	A88	A35	A28	A79	IM	nde %
D9	105/112	158	211/217	97/99	147	109	128/135	102/104	183/185	0.016
D10	105/112	158/160	211/217	97	147/149	91/109	135	102/104	183	0.016
D11	105/116	160/166	211/217	97	147	109	128/135	102/104	183/185	0.016
D12	110/112	158/166	211/217	97/99	147	91/109	135	90/104	183	0.016
D13	110/116	158/168	211	97	145/147	91/109	128/135	90/104	183/185	0.008
D14	112	158	211/217	97/99	145/147	91/109	135	102/104	183	0.031
D15	112	158/166	217	97/99	147/149	109	135	90/106	185	0.063
D16	112/116	158	211/217	97	147/149	109	128/135	90/106	183/185	0.016

Table 2 Number of matings of drone colonies in 1999 (D1–D8) and 2000 (D9–D16) with the test queens (Q1/99–Q6/99 for 1999, Q1/00–Q13/00 for 2000). Also shown are the numbers of offspring workers genotyped (n), observed numbers of matings (d_0), estimated number of matings (d_e) and the relative numbers of matings(ΔM in % as deviation from population mean mating frequency) of the drone colonies.

Queen	n	d_0	d_e	D1	D2	D3	D4	D5	D6	D7	D8
Q1/99	20	8	8.8	1.00	0	1.00	0.50	1.75	0.25	0.75	2.75
Q2/99	20	9	10.4	0	3.00	0	1.33	2.33	0.33	2.00	0
Q3/99	20	11	14.4	0.33	0.50	2.83	1.83	1.83	0.33	2.33	1.00
Q4/99	20	8	8.8	0	0.50	1.50	0.50	1.25	0.25	2.75	1.25
Q5/99	20	9	10.4	0	1.00	0	0.83	3.92	0.58	1.58	1.08
Q6/99	20	9	10.4	1.00	1.50	0.5	0.50	1.50	0	3.00	1.00
$\sum$	120	54	63.2	2.33	6.5	5.83	5.49	12.58	1.74	12.41	7.08
ΔM				−8.18	−0.45	−1.69	−2.32	10.81	−9.27	10.49	0.62
				D9	D10	D11	D12	D13	D14	D15	D16
Q7/00	28	5	5.0	0	1.00	0	1.00	1.00	0	0	2.00
Q8/00	28	4	4.0	0	0	0	1.00	0.50	1.00	1.50	0
Q9/00	30	9	9.3	2.00	1.00	0	0	1.00	2.00	1.00	2.00
Q10/00	28	3	3.0	0	0	0	1.00	2.00	0	0	0
Q11/00	22	3	3.0	0	0	0	1.00	1.00	0	1.00	0
Q12/00	30	9	9.3	0	0.50	0	3.50	1.00	4.00	0	0
Q13/00	28	9	9.3	0	0	0	2.00	2.00	1.00	2.00	2.00
Q14/00	30	5	5.0	1.08	1.08	0.25	1.60	0	1.00	0	0
Q15/00	27	9	9.3	2.00	0	0.50	0	3.00	0.50	2.50	0.50
Q16/00	24	5	5.0	0	0	0	0	2.50	0	0.50	2.00
Q17/00	25	13	14.4	1.00	0	0	5.00	1.5	5.00	0.5	0
Q18/00	27	8	8.2	0	0	1.50	0.50	0	2.50	2.50	1.00
Q19/00	26	11	11.7	0	1.00	0.50	1.00	0	3.50	2.50	2.50
$\sum$	362	93	118	6.08	4.58	2.75	17.6	15.5	20.5	14	12
ΔM				−5.96	−7.58	−9.54	6.42	4.17	9.54	2.55	0.40

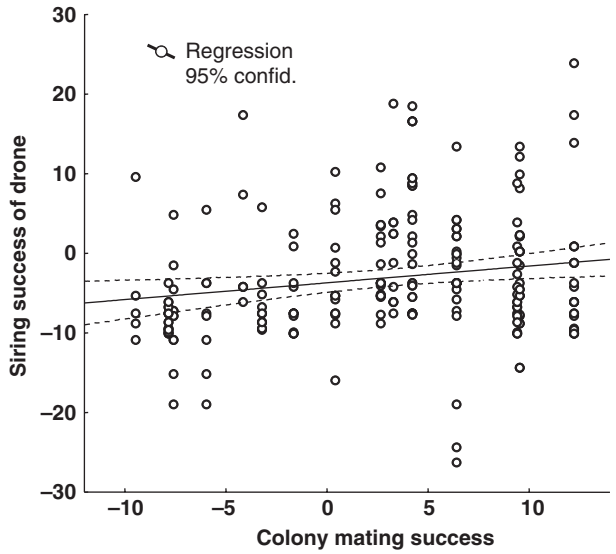

Fig. 2. Correlation between the number of matings per drone colony (presented as % difference to population mean) and the relative siring success (s) per individual drone (in % difference from mean). The regression line is given for illustrative purposes only, the significance was assessed by a Mantel test ($r = 0.49$; $P = 0.02$).

d_e ranged from 3.01 to 15.7 matings per test queen with a mean of 8.25 ± .3.61. The estimated number of matings was only marginally higher than the observed number of matings, indicating that most of the patrilines had been detected in the worker samples (Table 2).

The differences in the number of matings among the drone colonies were significant for both years (for 1999: $\chi^2 = 16.77$, d.f. = 7, $P < 0.02$; for 2000: $\chi^2 = 54.96$, d.f. = 7, $P < 0.0001$; Table 2). Figure 2 shows the relative siring success (s) of individual drones depending on the number of matings of the corresponding mother colonies. To overcome interdependence of the data sets we performed a Mantel test (Mantel, 1967; Sokal, 1979) on the relative siring success (s) of drones and the number of matings per colony using the software program *Mantel* (Liedloff, 1999). The Mantel test revealed a significant correlation between the relative siring success s of drones and the number of matings of the drone colonies (number of iterations = 5000; $r = 0.49$; $P = 0.02$).

Discussion

We found clear evidence for an extensive diversity in male mating success at the colony level. Some colonies had significantly more matings (about an order of magnitude) than other drone producing colonies in the same year. This is remarkable, because we had taken great care to standardize conditions for the drone producing colonies. All drone mothers were sister queens, all host colonies were of equal size and all had similar numbers of drones in the colony (two covered frames). Moreover, we found a significant positive correlation between the number of successfully mating drones per colony and the individual siring success of drones of these colonies. Thus drones of a 'fit' colony not only had a higher probability to mate with a queen but also the number of offspring sired in the mated queen was higher than that of its competitors. As the overall male fitness of a colony is the product of its mating success (the number of drones mating successfully) and the average siring success of these drones the differences between the drone colonies are even enhanced. Thus the differences among the drone colonies for the number of sired worker offspring are even more striking than the differences in matings alone (for 1999: $\chi^2 = 48.8$, d.f. = 7, $P < 0.0001$; for 2000: $\chi^2 = 67.47$, d.f. = 7, $P < 0.0001$). These huge differences in patriline frequencies by far exceed potential biases due to nonrandom sperm usage. Fluctuations in patriline frequencies within a colony are much smaller than observed in this study (Page & Metcalf, 1982; Haberl & Tautz, 1998).

So far it has been assumed that the number of drones rather than their individual mating success was most significant for colony male success (Baudry *et al.*, 1998). Moreover, the main selective force at the colonial level was considered to be on the female side (Seeley, 1985). The numbers of successful swarms produced has been stressed to be most important for natural selection at the colony level (Moritz & Southwick, 1992). Yet if we look at our present results, male success varied by an order of magnitude even under conditions where we experimentally equalized differences among the drones (by using sister mothers) and the drone producing colonies (by using equal sized colonies and numbers of drones per colony)! This suggests that under natural conditions, where colony sizes vary, male reproductive success is expected to vary even more and is very important for natural selection.

As not only the number of mating males but also the individual siring success of a drone is determined by the colony and/or the genotype of the mother queen, selection through the male side appears to be an extremely important factor for colony fitness. A successful drone colony does not receive any fitness gains through the workers or drones produced in an offspring colony (drones have no fathers). It does however gain fitness through the proportion of its patrilines in offspring colony as it raises the probability for rearing a daughter queen.

In our experiment the 'fit' colonies seem to have produced males which not only out-competed males from other colonies in mating, but also were more successful in post-mating competition. The fit colonies produced drones with a higher reproductive success than those of the other, less successful colonies. We do not know which effect caused the increased success of drones

from specific colonies. The amount of semen per drone is known to be highly variable (Woyke, 1973) and could be determined genetically and/or by the quality of the mother colony. Also the mating strategy of the individual drone could affect its success. Moritz (1986) showed for multiply inseminated queens a weak last male advantage. The last semen with which a queen was inseminated, had a higher frequency in the offspring than early inseminations. Given that both mechanisms (semen number and mating strategy) play a role, we suggest that the first may have a more prominent impact simply because of the much larger variance for semen number among drones reported so far. The amount of semen can vary by an order of magnitude whereas the differences in siring frequency from the first to the last semen load in an insemination only differed by 50% (Moritz, 1986). Also the quality of the semen can be highly important. Even if a high amount of sperm is transferred into the spermatheca, this does not necessarily imply that many offspring workers are sired.

This may be a highly relevant process to natural selection of honeybee populations at the colony level in addition to sex allocation bias which is a common phenomenon in social insect colonies (Crozier & Pamilo, 1996). Honeybee colonies can decide how much to invest in either sex based on local, environmental factors. Usually the drones (2000–5000 individuals per season) outnumber the queens (2–3 per season) produced by a colony by far, but a colony has to produce enough workers to facilitate reproductive swarming, which is also a major investment in queens. Weak colonies are known to produce very small numbers of drones or abandon drone rearing altogether (Gary, 1992). Also the number of colony fissions per season is correlated with the size and health condition of a colony. Whether colonies producing fitter drones invest less in the production of queens and daughter colonies cannot be answered from our data, because the drone producing colonies were artificially prevented from swarming, to avoid the weakening of the colonies.

Although the actual mechanism for this post-mating selection in our study must remain open, this is to our knowledge the first evidence for differences in the male reproductive success for honeybees in a natural mating situation.

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