SHORT COMMUNICATION

**Robin F. A. Moritz · H. Michael G. Lattorff ·
Peter Neumann · F. Bernhard Kraus ·
Sarah E. Radloff · H. Randall Hepburn**

Rare royal families in honeybees, *Apis mellifera*

Abstract The queen is the dominant female in the honeybee colony, *Apis mellifera*, and controls reproduction. Queen larvae are selected by the workers and are fed a special diet (royal jelly), which determines caste. Because queens mate with many males a large number of subfamilies coexist in the colony. As a consequence, there is a considerable potential for conflict among the subfamilies over queen rearing. Here we show that honeybee queens are not reared at random but are preferentially reared from rare "royal" subfamilies, which have extremely low frequencies in the colony's worker force but a high frequency in the queens reared.

Introduction

The honeybee queen, *Apis mellifera*, mates with extremely large numbers of males resulting in up to 45 subfamilies that coexist in the colony (Palmer and Oldroyd 2000). In temperate climates each queen produces only few reproductive swarms in her lifetime (Moritz and Southwick 1992), which

R. F. A. Moritz (✉) · H. M. G. Lattorff · P. Neumann ·
F. B. Kraus
Institut für Zoologie, Molekulare Ökologie,
Martin-Luther-Universität Halle-Wittenberg,
Hoher Weg 4,
06099 Halle Saale, Germany
e-mail: r.moritz@zoologie.uni-halle.de
Tel.: +49-345-5526223
Fax: +49-345-5527264

S. E. Radloff
Department of Zoology and Entomology, Rhodes University,
6140 Grahamstown, South Africa

H. R. Hepburn
Department of Statistics, Rhodes University,
6140 Grahamstown, South Africa

Present address:
F. B. Kraus
El Colegio de la Frontera Sur, Departamento Entomología
Tropical,
30700 Tapachula, Chiapas, México

is equivalent to the number of newly produced reproductive queens. The large number of subfamilies in the colony (Moritz et al. 1994) usually exceeds the small number of reproductive queens by far. As a consequence, kin selection theory (Hamilton 1964) predicts severe conflict among the workers resulting from competitive nepotistic rearing of queens from their own subfamilies. However, most attempts to demonstrate nepotistic queen rearing yielded either negative evidence (Breed et al. 1984; Woyciechowski 1990; Tilley and Oldroyd 1997) or statistically unsupported results (Page et al. 1989; Carlin and Frumhoff 1990; Oldroyd et al. 1990). Nevertheless, rearing of queens does not appear to be random (Estoup et al. 1994; Moritz et al. 1996; Tilley and Oldroyd 1997; Schneider and DeGrandi-Hoffmann 2003) and an individual from a specific subfamily may have a higher probability of becoming a queen than individuals from others. This is surprising, because natural selection should result in a low genetic variance for reproductive potential and equal chances for all subfamilies to produce a queen. Balanced selection has been asserted to explain the phenomenon (Moritz 1989; Oldroyd et al. 1992; Tilley and Oldroyd 1997; Montague and Oldroyd 1998), where individual fitness is gained at the expense of fitness at the colony level (Tarpy et al. 2004). We here present a case where the increased probability of becoming a queen can be plausibly explained based on individual direct fitness alone, resulting from a trade-off between male and female fitness. This results in a peculiar genetic composition of the colony, where individuals from "royal" families are extremely rare in the worker force but very frequent in the newly reared queens.

Materials and methods

Worker brood samples were collected from three *A. m. capensis* colonies (n=261) at Rhodes University, Grahamstown (South Africa) and five *A. m. carnica* colonies (n=221) at the Martin-Luther-University Halle-Wittenberg (Germany). Subfamily frequencies and distributions were determined by genotyping worker brood

Table 1 Results of the statistical tests to evaluate the difference between queen and worker subfamilies in the eight colonies

Colony	N_W	N_Q	N_{total}	k_o	k_e	p_{MC}
1	48	7	55	27	33.2	0.01904±0.00043
2	48	7	55	10	10	0.00627±0.00025
3	48	32	80	29	31.3	0.00096±0.0001
4	47	89	136	29	29.3	0.00662±0.00026
5	30	9	39	24	36	0.00059±0.00008
6	94	10	104	59	81.7	$<10^{-6}$
7	86	10	96	46	55.9	0.00001±0.00001
8	81	12	93	34	36.8	$<10^{-6}$
Total	482	176	658			$<10^{-6}$

N_W: sample size workers, N_Q: sample size queens, N_{total}: total sample size, k_o: observed subfamilies, k_e = estimated subfamilies, p_{MC}: p value from a Monte Carlo simulation for comparison of worker- and queen-subfamily frequency distributions of the pentiles

Table 2 Fitness values and frequencies of queens and males in a population with a locus that determines rearing female larvae to queens

Queens			Males			Offspring		
Genotype	Fitness	Frq	Genotype	Fitness	Frq	rr	$r+$	$++$
rr	1	p^2	r	$1-s$	p	1	0	0
	1	p^2	$+$	1	q	0	1	0
$r+$	1	2pq	r	$1-s$	p	1	0	0
	1	2pq	$+$	1	q	0	1	0
$++$	1	q^2	r	$1-s$	p	0	1	0
	1	q^2	$+$	1	q	0	0	1

$++$ Genotypes are only reared if no $r+$ or rr genotype is in the colony. The $r+$ genotypes are only reared if no rr genotype is present. The rr-genotypes are the only larvae reared to queens, if they are produced in a mating type. p: frequency r allele, q: frequency $+$ allele, s: selection coefficient

samples with at least eight polymorphic microsatellite DNA loci (Solignac et al. 2003). The colonies were dequeened to stimulate emergency queen rearing. After 14 days 32 and 144 queen pupae were sampled from the *A. m. capensis* and the *A. m. carnica* colonies respectively. The subfamily frequencies in the queen samples were determined with the same set of loci as used for the workers. Based on their genotype queen larvae were assigned to a corresponding worker subfamily if possible. We used a standard procedure (Cornuet and Aries 1980) to correct for potential sampling bias on the number of subfamilies (estimated subfamilies).

We grouped the subfamilies for each colony in pentiles with one fifth of the number of subfamilies in each group. This allowed for the comparison of colonies with unequal numbers of subfamilies. For each of these pentile groups we recorded the frequency of individual workers and queens. Differences between the frequency distributions were tested using the Monte Carlo R×C 2.21 program (WR Engels, University of Wisconsin, http://engels.genetics.wisc.edu/pstat/) with 100,000 replications.

The male haploid population genetic model for testing the trade-off hypothesis between male and female fitness was as follows: Given a single locus with two alleles, one making larvae attractive for queen rearing (*r*) and the other one not (+), a generation matrix (Table 2) can be constructed to test if stable equilibria between *r* and + can occur in an otherwise classical Hardy–Weinberg population. In the model we assume that the fitness of *r* males is reduced by the selection coefficient (*s* W_m=1-*s*). We also assume the extreme case, in which only those female larvae with the largest number of *r* alleles will invariably be raised to the queens in the colony. Thus, *rr* queen larvae are always preferentially reared over *r+* larvae, which in turn are preferred over ++ larvae. The fitness of adult queens is equal among all genotypes (W_f=1). Any other scenario with variable or partial fitness advantages of queen larvae genotypes relaxes the selection assumptions and enhances the parameter space in which stable equilibria can occur. Using these parameter settings (Table 2), the equilibrium frequency can be derived as

$$p = (1/s) - 1 \text{ (for } 0 < p < 1) \tag{1}$$

which results in stable equilibria between both alleles for 0.5<s<1. Allele *r* will go to fixation for s≤0.5 under these conditions (Fig. 1a). Other conditions for the rearing of queen larvae than those listed in Table 2 allow for many other possible equilibria conditions, showing that there is a vast parameter space where the *r* allele will neither go towards fixation nor extinction if there is a fitness trade-off between male and female sex.

Results

In eight colonies, the genotypes of the tested worker (*n*=482) and queen pupae (*n*=176) revealed between 10 and 59 subfamilies per colony. Several subfamilies (*n*=45 of 258) of the eight colonies exclusively contributed to
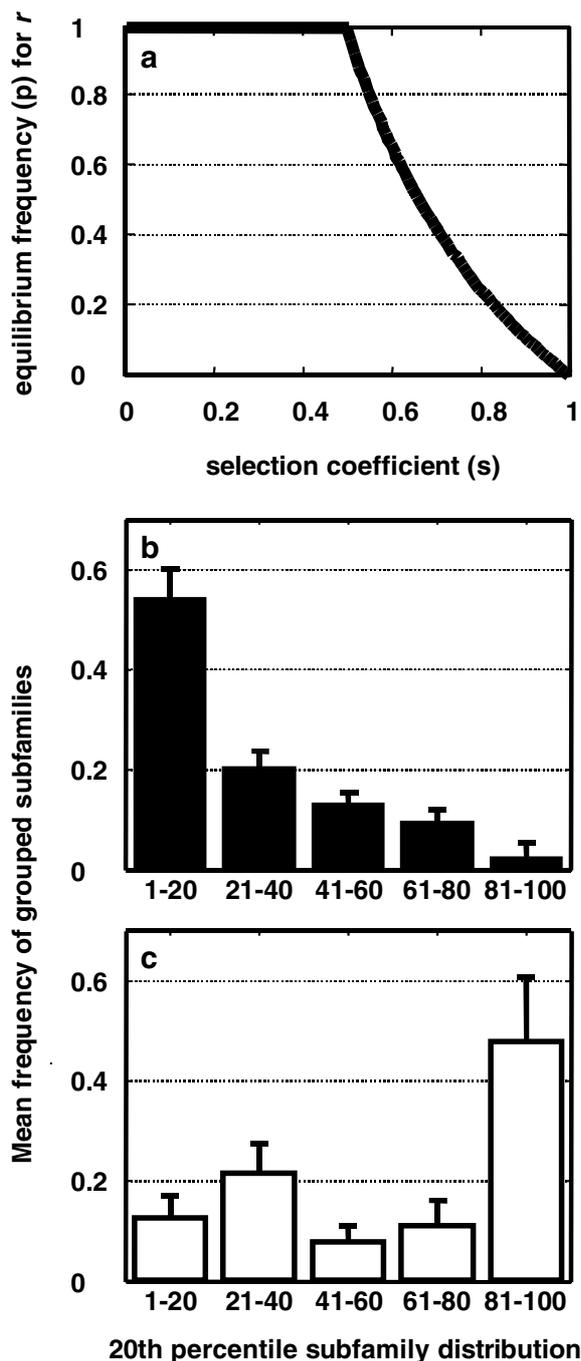
Fig. 1 **a** The equilibrium frequency of the royal allele *r* as predicted by the population genetic model. The equilibrium frequency of the *r* allele is $p=1$ for selection coefficients below $s=0.5$. Increasing the selection coefficient above $s=0.5$ reduces the frequency of the *r* allele towards $p=0$. **b** Frequency distribution for 20th percentiles of worker subfamilies. The distribution was derived from subfamilies ordered in descending frequency. Bars represent mean plus standard errors ($N=8$ colonies). While 20% of the most frequent subfamilies produce more than 50% of the workers on average, the 20% least frequent subfamilies produce only 3% of the worker force. **c** Frequency distribution for 20th percentiles of queen subfamilies. Frequencies were recorded according to the subfamily grouping applied to the worker sample. The 20% least frequent families in the worker sample were most frequent (up to 100%) in the queen sample

the queen sample and were not found in the worker sample. All individuals could be unambiguously identified as offspring of the resident queens. The subfamily distributions were significantly different between the queen and worker samples in all eight colonies (Table 1) suggesting that intracolonial selection processes had influenced with queen rearing. Queens are preferentially reared from the subfamilies, which are uncommon in the worker sample (top pentile, Fig. 1b, 1c). The strong positive correlation between the degree of polyandry and the number of rare subfamilies suggests that in colonies headed by queens with high mating frequencies, the phenomenon should be more explicit. Indeed, we find a positive correlation ($r=0.86$; $N=8$; $p=0.006$) when calculating the contribution of the top pentile subfamilies (the subfamilies which are rare in the worker sample) to the whole sample of emergency queens relative to the mating frequency. In contrast there is no such correlation for the worker sample ($r=0.03$; $N=8$; $p=0.939$). Because the effect of rare royal subfamilies is more pronounced in colonies composed of a large number of subfamilies, it seems likely that previous studies did not detect this effect due to relatively small numbers of subfamilies in the colonies (Page et al. 1989).

Discussion

The vast majority of emergency queens were reared from subfamilies that remained undetected in the worker sample or had a low frequency and are therefore rare by definition. Under a random queen rearing regime the subfamily distribution in queens should not be different from the worker sample. Nepotistic queen rearing can hardly explain our results, because many workers are required to rear a queen and repeated visits of nurse bees are essential to maintain the high nutritional demand of developing queen larvae. Therefore, it seems unlikely (though not impossible) that the few workers from rare subfamilies recruit themselves throughout the colony to jointly develop into nurse bees, seek the queen cells, and only invest into their own super-sisters. In addition, such a mechanism is unlikely because intracolonial kin-recognition could not empirically be shown for queen rearing in the honeybee, in spite of repeated efforts (Breed et al. 1994).

Because the subfamily composition in the queens is different from that of the worker larvae, it seems plausible to assume that those larvae, which were reared to queens, were particularly attractive to the nurse bees rearing them. Genetic variance for rearing attractiveness of larvae has been shown several times in honeybees (Tilley and Oldroyd 1997; Beekman et al. 2000; Calis et al. 2002; Franck et al. 2002; Schneider and DeGrandi-Hoffmann 2003). In all genetic studies, the most frequent subfamilies in the worker sample were under-represented and rare subfamilies were over-represented in the queen sample (although not as strikingly as in our case). If larvae from rare subfamilies are preferentially reared to queens there

are two possible explanations. The first is that workers prefer to rear uncommon genotypes to queens in a frequency dependent selection process. Although we cannot exclude this mechanism, there is neither theoretical reasoning nor empirical evidence in support of such a mechanism. The second explanation is based on variation in reproductive traits among the fathering drones. Semen fertility and number of spermatozoa of the siring drone have been shown to be important for male fitness (Schlüns et al. 2004). Indeed, fitness among honeybee males can vary in excess of an order of magnitude (Kraus et al. 2003). In our study males with few offspring in the workers were those producing the vast majority of reproductive females. This might indicate a trade-off between male and female individual fitness. If males, which contribute less semen or have less fertile semen, are those producing more attractive larvae for queen rearing, this results in an increased fitness for female sexuals. A population genetic model (Table 2) shows that stable equilibria can occur over a wide fitness parameter space for male and female sexuals (details of the model are given within the methods section). Such a trade-off between male and female fitness has recently been found in other insects (Chippindale et al. 2001; Fedorka and Mousseau 2004) and serves well as a plausible explanation for the observed rearing pattern of emergency queens in honeybee colonies. We cannot exclude that emergency queen rearing principally differs form the rearing of queens in preparation of reproductive swarming. Under these conditions the queens lays eggs into specifically constructed queen rearing cells and hence the distribution of subfamilies of swarm queens may not be identical to emergency queen rearing. However, if our model reflects the actual proximate mechanism for the selection of queen larvae in honeybee colonies, this would give rise to paternal lineages of royal families, which indeed are only rarely found in the worker caste.

References

Beekman M, Calis JNM, Boot WJ (2000) Parasitic honeybees get royal treatment. Nature 404:723

Breed MD, Velthuis HHW, Robinson GE (1984) Do worker honey bees discriminate among related and unrelated larval phenotypes? Ann Entomol Soc Am 77:737–739

Breed MD, Welch CK, Cruz RC (1994) Kin discrimination within honey bee (*Apis mellifera*) colonies: an analysis of the evidence. Behav Proc 33:25–40

Calis JNM, Boot WJ, Allsopp MH, Beekman M (2002) Getting more than a fair share: nutrition of worker larvae related to social parasitism in the Cape honeybee *Apis mellifera capensis*. Apidologie 33:193–202

Carlin NF, Frumhoff PC (1990) Nepotism in the honeybee. Nature 346:707–708.

Chippindale AK, Gibson JR, Rice WR (2001) Negative genetic correlation for adult fitness between sexes reveals ontogenetic conflict in *Drosophila*. Proc Natl Acad Sci USA 98:1671–1675

Cornuet JM, Aries F (1980) Number of sex alleles in a sample of honeybee colonies. Apidologie 11:87–93

Estoup A, Solignac M, Cornuet JM (1994) Precise assessment of the number of patrilines and of genetic relatedness in honey bee colonies. Proc Roy Soc Lond Ser B 258:1–7

Fedorka KM, Mousseau TA (2004) Female mating bias results in conflicting sex-specific offspring fitness. Nature 429:65–67

Franck P, Solignac M, Vautrin D, Cornuet JM, Koeniger G, Koeniger N (2002) Sperm competition and last-male precedence in the honeybee. Anim Behav 64:503–509.

Hamilton WD (1964) The genetical evolution of social behaviour, I & II. J Theor Biol 7:1–52

Kraus FB, Neumann P, Scharpenberg H, van Praagh J, Moritz RFA (2003) Male fitness of honeybee colonies (*Apis mellifera* L.). J Evol Biol 16:914–920

Montague CE, Oldroyd BP (1998) The evolution of worker sterility in honey bees: An investigation into a behavioural mutant causing failure of worker policing. Evolution 52:1408–1415

Moritz RFA, Southwick EE (1992) Bees as superorganisms. An evolutionary reality. Springer, Heidelberg

Moritz RFA (1989) Colony level and within colony level selection in honeybees. A two allele population model for *Apis mellifera capensis*. Behav Ecol Sociobiol 25:437–444

Moritz RFA, Kryger P, Allsopp MH (1996) Competition for royalty in bees. Nature 384:31

Moritz RFA, Kryger P, Koeniger G, Koeniger N, Estoup A, Tingek S (1994) High-degree of polyandry in *Apis dorsata* queens detected by DNA microsatellite variability. Behav Ecol Sociobiol 37:357–363

Oldroyd BP, Rinderer TE, Buco SM (1990) Nepotism in the honeybee. Nature 346:707–708

Oldroyd BP, Rinderer TE, Harbo JR, Buco SM (1992) Effects of intracolonial genetic diversity on honey bee (Hymenoptera: Apidae) colony performance. Ann Entomol Soc Am 85:335–343

Page RE Robinson GE, Fondrk MK (1989) Genetic specialists, kin recognition, and nepotism in honey-bee colonies. Nature 338:576–579

Palmer KA, Oldroyd BP (2000) Evolution of multiple mating in the genus Apis. Apidologie 31:235–248

Schlüns H, Koeniger G, Koeniger N, Moritz RFA (2004) Sperm utilization pattern in the honeybee (*Apis mellifera*). Behav Ecol Sociobiol 56:458–463

Schneider SS, DeGrandi-Hoffmann G (2003) The influence of paternity on virgin queen success in hybrid colonies of European and African honeybees. Anim Behav 65:883–892

Solignac M, Vautrin D, Loiseau A, Mougel F, Baudry E, Estoup A, Garnery L, Haberl M, Cornuet JM (2003) Five hundred and fifty microsatellite markers for the study of the honeybee (*Apis mellifera* L.) genome. Mol Ecol Notes 3:307–311

Tarpy DR, Gilley DC, Seeley TD (2004) Levels of selection in a social insect:a review of conflict and cooperation during honey bee (*Apis mellifera*) queen replacement. Behav Ecol Sociobiol 55:513–523

Tilley CA, Oldroyd BP (1997) Unequal subfamily proportions among honey bee queen and worker brood. Anim Behav 54:1483–1490

Woyciechowski M (1990) Do honey bee, *Apis mellifera* L., workers favour sibling eggs and larvae in queen rearing? Anim Behav 39:1220–1222