ANIMAL BEHAVIOUR, 2004, **67**, 681–688
doi:10.1016/j.anbehav.2003.08.004

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Differences in nestmate recognition for drones and workers in the honeybee, *Apis mellifera* (L.)

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(Received 13 May 2003; initial acceptance 26 June 2003; final acceptance 4 August 2003; MS. number: 7723)

Nestmate recognition is the basic mechanism for rejecting foreign individuals and is essential for maintaining colony integrity in insect societies. However, in honeybees, *Apis mellifera*, both workers and males occasionally gain access to foreign colonies in spite of nest guards (=drifting). Instead of conducting direct behavioural observations, we inferred nestmate recognition for males and workers from the genotypes of naturally drifting individuals in honeybee colonies. We evaluated the degree of polyandry of the resident queens, because nestmate recognition theory predicts that the genotypic composition of insect colonies may affect the recognition precision of guards. Workers ($N = 1346$) and drones ($N = 407$) from 38 colonies were genotyped using four DNA microsatellite loci. Foreign bees were identified by maternity testing. The proportion of foreign individuals in a host colony was defined as immigration. Putative mother queens were identified if a queen's genotype corresponded with the genotype of a drifted individual. The proportion of a colony's individuals in the total number of drifted individuals was defined as emigration. Drones immigrated significantly more frequently than workers. The impact of polyandry was significantly different between drones and workers. Whereas drones immigrated more readily into less polyandrous colonies, worker immigration was not correlated with the degree of polyandry of the host colony. Furthermore, colonies with high levels of emigrated drones did not show high levels of emigration for workers, and colonies that adopted many workers did not adopt many foreign drones. Our data indicate that genetically derived odour cues are important for honeybee nestmate recognition in drones and show that different nestmate recognition mechanisms are used to identify drones and workers.

Nestmate recognition is predicted by inclusive fitness theory (Hamilton 1964a, b) and well developed in many social insect species (Crozier & Pamilo 1996). Foreign individuals are either rejected or accepted by guarding individuals based on a comparison of odour labels with a recognition template (Lacy & Sherman 1983). The cues involved in label and template formation are of genetic and/or environmental origin in a variety of species (Wallis 1962; Mabelis 1979; Carlin & Hölldobler 1986; Stuart 1987; Waldman et al. 1988; Pirk et al. 2001). Recognition in many taxa seems to be mediated by genetic-cue diversity (Ratnieks 1991), and genetically based mechanisms have been most lucidly illustrated by the classical work on the sweat bee *Lasioglossum zephyrum* (Kukuk et al. 1977; Greenberg 1979). Sweat bees are accepted into the nest based on the genetic relatedness between intruder and guard. However, in other species, recognition appears to be primarily environmentally determined (Ratnieks 1991), probably because environmental cues (Bethe 1898; Crosland 1989; Heinze et al. 1996; Liang & Silverman 2000) override genetically based ones (Breed 1983; Gamboa et al. 1986; Bennett 1989; Hölldobler & Wilson 1990; Beye et al. 1997, 1998). Clearly, nestmate recognition relies on both genetic and environmental compounds to a varying extent (Wilson 1971; Jutsum et al. 1979; Breed 1983; Carlin & Hölldobler 1986; Crozier 1988; Pirk et al. 2001). Indeed, even within a single species, populations may differ significantly with respect to the relative importance of environmental and genetic cues (e.g. *Formica pratensis*: Pirk et al. 2001). The proximate cues seem to be cuticular hydrocarbons, which function as nestmate recognition cues in a variety of social insect species (e.g. Wagner et al. 2000; Fröhlich et al. 2000, 2001; Ruther et al. 2002; Lavine et al. 2003).

The vast majority of nestmate recognition studies have addressed nestmate recognition for workers. This seems plausible because workers are the guarding individuals accepting or rejecting foreign individuals, whereas the female and male sexuals usually do not participate in such activities. Furthermore, workers are easily accessible at the

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0003–3472/03/$30.00/0

nest surface during the entire field season, whereas sexuals are usually present for only brief periods. However, nestmate recognition for male and female sexuals can be more important than for workers. The acceptance of unrelated female reproductives clearly represents a fitness disadvantage for the host colonies. For example, this is the case in intraspecific social parasitism (Neumann & Moritz 2002) or in polygynous ant species (Hölldobler & Wilson 1990). Nestmate recognition for males can be important for avoiding incest (Ryan & Gamboa 1986; Foster 1992) or for reducing costs to the host of adopting foreign males (male reproductive parasitism, Rinderer et al. 1985).

Although few studies have addressed nestmate recognition for male or female sexuals (e.g. Smith 1983; Shellman-Reeve & Gamboa 1985; Sundström 1997; Van der Meer & Porter 2001), there is evidence of fundamental differences between the nestmate recognition mechanisms for workers and those for queens and males. For example, queens and workers may differ significantly in their chemical profiles, suggesting that the two castes bear specific labels (Tentschert et al. 2002). Similarly, discriminant analyses of the cuticular lipid profiles of adult wasps (*Polistes metricus*) showed that male compound composition is significantly different from that of females (Layton et al. 1994). In some cases, nestmate recognition abilities were low for female sexuals (Brown et al. 2003). On the other hand, there seems to be a sex-ratio-dependent execution of queens in polygynous colonies (Brown et al. 2003). Furthermore, genetic data suggest that related females are primarily adopted after their mating flights in polygynous ant colonies (*F. sanguinea*: Pamilo & Varvio-Aho 1979). Evidence from the fire ant, *Solenopsis invicta*, strongly suggests that nestmate recognition involving female sexuals does not follow the same mechanism used to explain nestmate recognition behaviour between workers (Van der Meer & Porter 2001). Nestmate recognition for males has been documented in *L. zephyrum* (Smith 1983) and in polistine wasps (Shellman-Reeve & Gamboa 1985).

To study potential differences in nestmate recognition between workers and males, the western honeybee, *A. mellifera*, appears to be a prime test system. Honeybee males, drones, are produced in large numbers and perform numerous flights. Upon each return, the drones undergo a recognition event testing the mechanism. Furthermore, the honeybee queen shows an exceptionally high degree of polyandry (Moritz et al. 1995; Neumann & Moritz 2000; Palmer & Oldroyd 2000) with up to 45 matings for *A. mellifera* (Moritz et al. 1996), yielding a large variance for genotypic colony composition. Finally, colonies have highly specialized guard bees that scrutinize incoming bees (e.g. Breed 1983; Breed & Julian 1992) and either reject or accept individuals. Nevertheless, drones and workers from foreign colonies can be adopted as new nest members (Breed et al. 1992). This drifting of honeybees requires two independent behavioural mechanisms (Moritz & Neumann 1996): (1) individual orientation errors of bees returning to the nest and (2) acceptance by the host colony thereby passing the guard's discrimination system. The latter mechanism, therefore, potentially serves as an excellent test system for nestmate recognition in honeybees in a natural set-up.

Under experimental conditions, honeybees can discriminate between nestmates and non-nestmates by genetic cues alone (Breed 1983; Getz & Smith 1983; Moritz & Hillesheim 1990a, b). In contrast, Downs & Ratnieks (1999) showed that under more natural conditions, workers use environmental cues to decide whether to accept an incoming honeybee to the colony. They concluded that heritable cues are not used in nestmate recognition. However, they studied the aggression of guard bees only towards infertile workers. From an inclusive fitness perspective, genetic relatedness should be unimportant for such a trait, because workers are usually infertile and therefore unlikely to reproduce in the guard bees' colony (however, see Neumann & Moritz 2002 for social parasitic workers). The prime risk of accepting non-nestmate workers into the colony lies in the potential danger of the intruder robbing the colony's honey stores. Regardless of relatedness, the workers should therefore reject all non-nestmate workers.

Drones are accepted more readily into foreign colonies and drifting of drones is a common phenomenon in apiaries (Goetze 1954; Washington 1967; Moritz & Neumann 1996; Neumann et al. 2000). Because honeybees mate in mid-air in drone congregation areas far away from the colony, incest avoidance in the nest is irrelevant (Ratnieks 1991). Drones are sexual reproductives, who are fed by the workers and may be costly for the colony (Rinderer et al. 1985). Guards might therefore accept foreign drones based on a recognition mechanism different from that for workers. We studied the effects of genotypic composition of both host and mother colony on the acceptance of foreign workers and drones in honeybee colonies, for which we used DNA microsatellite genotyping to identify native and drifted individuals.

METHODS

Experimental Design and Sampling

Samples were obtained from 38 queenright colonies of *A. m. carnica* at the apiary of the Bayerische Landesanstalt für Bienenzucht at Schwarzenau, Germany (Fig. 1; see also Neumann et al. 2000). Five unrelated colonies were each grouped in colony clusters, which enhanced drifting within a cluster (Kühl & Neumann 1996). At least 40 sexually mature drones and workers were sampled in colonies ($N = 13$), which had an ample drone supply (few hundreds). Adult workers ($N > 40$) were sampled per colony in 25 additional colonies, which had insufficient numbers of drones. All the samples were stored in 75% ethanol until DNA extraction.

DNA Extraction and Genotyping

DNA was extracted following routine protocols (Neumann et al. 1999a, b, c) and genotyped at four variable DNA microsatellite loci (A43, B124, A76, A107; Estoup et al. 1993, 1994; Solignac et al. 2003). We used Mendelian rules to infer queen and sire genotypes from worker genotypes. Individuals lacking putative mother

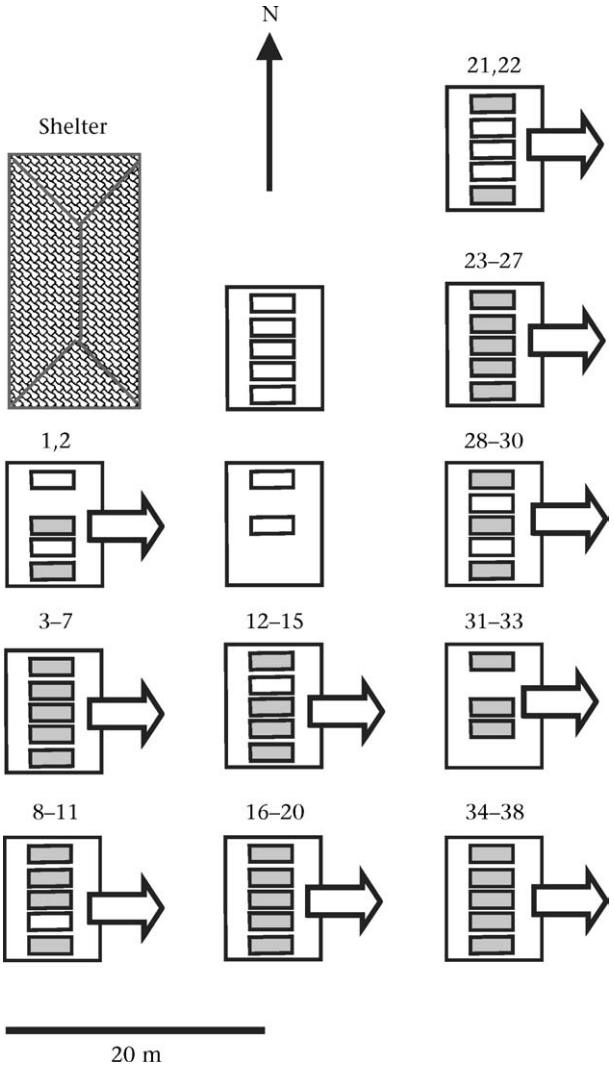

Figure 1. Location of the 38 test colonies in the apiary. Each sampled colony is indicated by a shaded rectangle (white rectangle = nonsampled colony). The colonies were grouped in clusters of five. The colony ID numbers are given above each cluster. All flight entrances faced east (open arrows).

queen alleles, at least at one of the loci tested, were considered to have drifted (Neumann et al. 1999a). The physical number of matings, k, was estimated from the observed number of matings (Cornuet & Aries 1980) to correct for the finite sample size.

Putative Mother Colonies of Drifted Individuals

The genotypes of the drifted individuals were assigned to the corresponding mother queens. If the genotype of a drifted drone corresponded to a potential gamete type of a queen, we considered it to be a son of that particular queen. When the genotype of a drifted worker corresponded to a queen genotype, we considered that worker to be the offspring of that specific queen. If the genotypes corresponded to more than one queen, the workers were assigned based on the potential drone fathers. Seven individuals with no corresponding genotypes (four drones and three workers) were excluded from further data analysis. Such individuals most likely originated from nonsampled colonies at the same apiary (Fig. 1).

Immigration and Emigration

The proportion of drifted individuals in a given colony i was defined as the immigration (I_i) into that particular host colony.

$$I_i = \frac{\sum_{j=1,\, j\neq i}^{m} n_{ij}}{\sum_{j=1,\, j\neq i}^{m} n_{ij} + n_{ii}} \qquad (1)$$

where n_{ij} = the number of foreign individuals of colony j sampled in host colony I, n_{ii} = the number of native individuals sampled in host colony I and m = total number of sampled colonies. The ratio between the total number of individuals of colony i in all foreign host colonies and the total number of all foreign individuals in host colonies was defined as the emigration (E_i) of a mother colony.

$$E_i = \frac{\sum_{j=1}^{m} n_{ji}}{\sum_{j=1}^{m}\sum_{k=1}^{m} n_{jk}} \qquad (2)$$

where n_{ji} = the number of individuals of colony i sampled in colony j, $\sum n_{jk}$ = the number of all foreign individuals sampled in colony j. E_i thus reflects the proportion of drifted individuals of a given mother colony i in relation to all drifted individuals in the sample. Assuming that all colonies are equally strong and that all individuals (drones or workers) are accepted with the same probability, the emigration value of a colony E_i is an estimate of the colony's contribution to the pool of drifting individuals. Colonies with a high emigration value contribute more to the drifters than colonies with a low E_i value.

Data Analysis

Drifted individuals may by chance share an allele with the host queen at all the loci tested and thus be misidentified as native. This nondetection error for foreign workers and drones was determined according to Neumann et al. (1999a).

To compare the immigration of workers and drones, we used a Mann–Whitney U test. Regressions of immigration and emigration of workers and drones on the number of estimated matings were computed. The slopes for drones and workers were compared and tested with a two-tailed t test. The statistical test procedures were performed with Statistica and the power analysis with GPOWER (Erdfelder et al. 1996).

RESULTS

From a total of 1753 genotyped individuals (1346 workers and 407 drones), 66 drifted workers and 227 drifted drones could be assigned to mother colonies (Table 1). The mean frequency of emigrated drones ± SE was 0.065 ± 0.020 (range 0.015–0.263). The mean immigration of drones ± SE was 0.570 ± 0.065 (range 0.242–0.929). The mean emigration of workers ± SE was 0.026 ± 0.005 (range 0–0.13) and the mean immigration ± SE was 0.049 ± 0.007 (range 0–0.15). The estimated average number of matings ± SE was 24.12 ± 1.76 (range 10.41–54.55), higher than the observed number of matings in the finite sample (17.71 ± 0.83).

The error for not detecting a drifted individual because it shared alleles of the host colony's queen by chance (nondetection error) was computed according to Boomsma & Ratnieks (1996) using the allele frequencies of the tested honeybee population (Table 2) and was 0.0043 for drones and 0.0194 for workers. Immigration was significantly higher in drones than in workers (Mann–Whitney U test: $U = 0$, $N_1 = 13$, $N_2 = 38$, $P < 0.0001$). There were no significant correlations between drone and worker immigration and/or emigration (Table 3). However, the correlation between immigration of drones and the estimated number of matings for polyandry of the host colony ($r_S = 0.56$, $P < 0.05$), as well as the estimates for the emigration of workers and polyandry of the mother colony ($r_S = 0.49$, $P < 0.01$), revealed significant differences (Table 3). The regression slope for worker emigration ($\beta_{E_{worker}} = 0.002$) is significantly steeper than that for drone emigration ($\beta_{E_{drone}} = 0.001$, $t_{47} = 2.41$, $P < 0.02$; Fig. 2a). The slope for drone immigration is

Table 1. Emigration and immigration of workers and drones and degree of polyandry of queens

	Drones (N=407)			Workers (N=1346)			Polyandry	
Colony$_i$	E_i	I_i	n	E_i	I_i	n	n_o	k
1				0.000	0.067	30	14	17.24
2				0.013	0.030	33	10	10.41
3	0.015	0.286	14	0.028	0.026	39	19	25.68
4	0.020	0.600	20	0.000	0.082	61	28	34.80
5				0.028	0.150	40	18	23.18
6	0.034	0.300	20	0.028	0.079	38	18	22.69
7	0.015	0.385	13	0.013	0.065	62	20	21.32
8				0.000	0.000	22	11	25.66
9				0.000	0.031	32	13	14.63
10				0.041	0.000	32	14	16.05
11				0.000	0.000	27	10	10.41
12				0.041	0.000	38	24	37.24
13				0.000	0.059	34	17	21.93
14				0.133	0.028	36	26	54.55
15				0.000	0.025	40	11	11.31
16				0.013	0.025	40	21	27.47
17	0.015	0.721	43	0.013	0.111	36	17	21.93
18	0.263	0.743	35	0.028	0.091	33	22	33.05
19	0.143	0.929	42	0.067	0.040	25	11	12.83
20	0.073	0.714	42	0.000	0.044	23	15	20.75
21				0.013	0.000	24	14	14.73
22				0.028	0.077	26	12	14.73
23				0.028	0.065	31	18	27.14
24				0.067	0.000	32	20	30.05
25				0.013	0.000	32	17	21.93
26				0.013	0.071	28	14	18.18
27				0.028	0.100	30	12	13.82
28				0.000	0.000	32	12	13.01
29				0.054	0.118	34	17	23.17
30				0.013	0.133	30	15	20.75
31				0.013	0.025	40	22	30.00
32				0.028	0.000	42	22	28.18
33				0.041	0.046	44	20	24.04
34	0.049	0.667	42	0.041	0.077	39	26	51.04
35	0.129	0.242	33	0.095	0.071	42	26	48.63
36	0.034	0.311	45	0.054	0.050	40	24	37.24
37	0.015	0.867	30	0.013	0.026	39	25	15.31
38	0.044	0.643	28	0.013	0.050	40	18	21.54
Mean	0.065	0.570		0.026	0.049		17.71	24.12
SE	0.020	0.065		0.005	0.007		0.83	1.76

E = emigration, I = immigration, n = sample size of genotyped individuals, n_o = number of observed matings, k = number of estimated matings (Neumann et al. 1999b, c; Neumann & Moritz 2000). Emigration was computed only for those colonies for which immigration data were available.

Table 2. Allele frequencies of the tested honeybee population (allele sizes in base pairs) based on the analysis of the sexual reproductives

Locus A76 ($N=743$)		Locus A107 ($N=753$)		Locus B124 ($N=739$)		Locus A43 ($N=715$)	
Allele (bp)	Frequency	Allele (bp)	Frequency	Allele (bp)	Frequency	Allele (bp)	Frequency
209	0.016	141	0.007	212	0.022	124	0.001
231	0.040	158	0.085	214	0.507	126	0.098
233	0.013	159	0.025	216	0.242	127	0.386
239	0.015	160	0.114	218	0.100	139	0.015
243	0.067	162	0.066	220	0.056	140	0.443
249	0.018	163	0.033	222	0.031	141	0.007
251	0.073	164	0.056	224	0.009	142	0.010
255	0.021	165	0.020	228	0.008	146	0.039
259	0.050	166	0.066	230	0.016		
261	0.044	167	0.039	232	0.001		
265	0.077	168	0.076	234	0.007		
267	0.051	169	0.009				
271	0.081	170	0.068				
277	0.028	171	0.045				
279	0.011	172	0.064				
281	0.011	173	0.015				
283	0.047	174	0.021				
287	0.047	175	0.042				
289	0.003	176	0.076				
291	0.015	177	0.053				
295	0.022	181	0.012				
299	0.059	183	0.007				
305	0.031	189	0.003				
311	0.020						
313	0.073						
325	0.007						
331	0.004						
343	0.037						
353	0.026						

Sample sizes (N = chromosomal sets) are not equal for all loci because not all loci could be used in all samples.

negative ($\beta_{I_{drone}} = -0.008$) and is significantly smaller than that for worker immigration ($\beta_{I_{worker}} = 0.0003$, $t_{47} = 2.57$, $P<0.02$; Fig. 2b).

DISCUSSION

Our study took advantage of an apiary layout as the basis for a field experiment, enhancing the potential for orientation errors and drifting, but without experimentally interfering with individual honeybee behaviour. The effects of the spatial arrangement of the colonies on drifting in the apiary have been addressed by Kühl & Neumann (1996) and the underlying drifting mechanisms are similar for workers and drones (Moritz & Neumann 1996). Our results presented here imply that nestmate recognition for drones is mediated by genetic-cue diversity. Genetic cues appeared to be less important for worker recognition. From an evolutionary perspective it may not be that surprising to observe different mechanisms for drones and workers. The consequences of accepting a foreign drone or a worker are very different for the colony. The colony should be extremely careful in adopting foreign workers, especially when nectar is scarce because these can be potential robber bees. Such robber bees steal honey and subsequently recruit more robbing workers from their natal colony, which may be lethal for the entire colony regardless of the relatedness between host colony and intruding worker. Relatedness is therefore unlikely to be important for worker acceptance, which may be reflected by the extremely low rate of adoption of nonrobbing workers. Foreign workers in our sample were most probably drifted workers who had been adopted in a new host colony rather than robbers. Once adopted, the workers become resident members of the new colony and usually do not return to their natal colony. Although these adopted workers show a reduced activity in the host colony (Neumann et al. 2003), they nevertheless enhance the colony's workforce to the benefit of the host.

The drones also impose a cost, but to a much lesser extent. Although they feed on the colony's supplies, they are not an immediate lethal threat to the colony. The costs are particularly low if the drones are related to the hosting colony and the workers gain indirect fitness. Relatedness is therefore more likely to play a role in recognition mechanisms for drones. Although drifting is the product of both orientation errors of individuals and their acceptance by the guard bees of the host colony, we cannot dissect these two behavioural components with our indirect experimental approach. Nevertheless, we can use our results to identify the number of immigrants in a colony as well as a colony's contribution to the total number of emigrating individuals.

Table 3. Correlation matrix (Spearman rank correlation, upper right) and power (lower left) for the immigration and emigration of workers and drones and the number of estimated matings *k*

		Emigration		Immigration	
	k	Drones	Workers	Drones	Workers
Number of estimated matings	—	0.14	0.49**	−0.56*	0.11
Emigration					
Drones	0.12	—	0.45	0.21	0.17
Workers	0.96	0.52	—	−0.23	0.14
Immigration					
Drones	0.74	0.18	0.20	—	−0.04
Workers	0.16	0.14	0.21	0.06	—

*$P<0.05$; **$P<0.001$.

Emigration

Emigration is a measure of individuals not returning to their mother colony but instead entering a foreign sampled colony. Emigration of workers was significantly correlated with the degree of polyandry in the mother colony. Workers of highly polyandrous colonies drifted significantly more often into foreign colonies than did workers of less polyandrous colonies. A returning worker uses odour cues to identify its home colony (von Frisch 1967). If the various father drones contribute to a common blend of labels, as part of a colony gestalt template (Crozier & Dix 1979), then drifting workers from highly polyandrous colonies returning to a foreign colony may be more prone to orientation errors than those of oligoandrous colonies. Workers from polyandrous colonies may have a more average odour template of their home colony. Lost workers may be less likely to recognize their orientation mistake at a foreign flight entrance (when comparing their template with the labels of the host colony) and thus more readily enter foreign colonies.

The emigration of drones showed no significant correlation with the estimated degree of polyandry. However, the sample size for the drones was considerably smaller than that for workers and the power of the test was low (0.12). Therefore, we cannot conclude whether the lack of significance is due to sample size alone or whether it reflects the true absence of this orientation mechanism in drones. The high numbers of drifted drones matches observations that drones in general are less precise than workers in returning to their home colonies (cf. Moritz & Neumann 1996).

Immigration

Immigration reflects the acceptance of alien individuals into a specific colony and is thus a measure of the scrutiny of the guard bees. Our data further support earlier studies that foreign drones are accepted at much higher frequencies than foreign workers (Goetze 1954; Witherell 1965; Washington 1967; Rinderer et al. 1985). There was a pronounced difference between worker and drone immigration, suggesting that sex- and caste-specific mechanisms or both are involved in honeybee nestmate

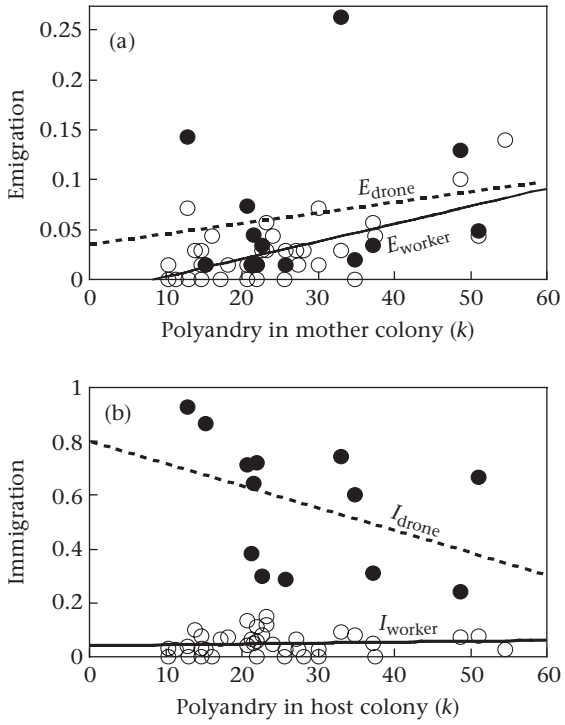

Figure 2. (a) Emigration of drones (●, E_{drone}) and workers (○, E_{worker}) from mother colonies with different degrees of polyandry into foreign host colonies. (b) Immigration of foreign drones (●, I_{drone}) and workers (○, I_{worker}) into host colonies with different degrees of polyandry.

recognition. A significant negative correlation is found between foreign drone immigration into a host colony and its degree of polyandry but such a correlation is lacking for workers. Workers were accepted in slightly higher proportions with increasing number of subfamilies in the colony but the slope was not significantly different from zero. The overall extremely low rate of worker immigration ($I_{worker} = 0.05$) suggests that foreign workers are either much more carefully scrutinized by the guards than drones ($I_{drone} = 0.57$) or make fewer attempts to enter foreign colonies. The guard bees of the more polyandrous colonies are obviously better at rejecting non-nestmate drones. One explanation could be that the guard bees use the full phenotypic complexity of the home colony cues as a template when assessing incoming drones, similar to the predictions made by the genotypic (or phenotypic) identity models (Pirk et al. 2001). Obviously, the guards' templates are not more average in the polyandrous colonies (as suggested for the orientation of returning foragers) but the genotypic complexity facilitates more accurate discrimination between native and foreign drones. Despite observations that the aggressive behaviour of guard bees towards foreign workers and drones can be similar (Kirchner & Gadagkar 1994), colonies seem to accept foreign drones and workers on the basis of very different nestmate recognition mechanisms. Although guards may scrutinize incoming workers much more carefully, they do this independently of genotypically derived cues, strongly supporting Downs & Ratnieks' (1999) findings.

Regulation of Foreign Drones and Workers

Colonies with high levels of emigrated drones did not show high levels of emigration for workers. Similarly, colonies that adopted many workers did not necessarily adopt many foreign drones. This further reveals the different mechanisms for nestmate recognition of drones and workers. Under the same adoption or rejection mechanism, one would expect a common trend for host colonies to accept foreign drones and workers. Although the odour blend of a colony is highly complex and environmental effects such as wax, pollen and nectar (Getz 1991; Breed 1998) certainly play a role, our results confirm the work of Breed (1983), who showed the significance of genetic factors for honeybee nestmate recognition. However, under the natural conditions in our study, this is valid only for drones, and workers are adopted on the basis of very different recognition mechanisms.

In conclusion, we recommend studying nestmate recognition for both sexes and across castes in more social insect species, because the underlying mechanisms seem to be fundamentally different and may shed new light on the evolution of kin and colony recognition in general.

Acknowledgments

We are grateful to K. Gladasch and A. Kühl for technical assistance and to D. Mautz for allowing us to take samples. We thank H. R. Hepburn and P. Kryger for useful comments on the manuscript. Financial support was granted by the DFG.

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