

Research article

Complex sociogenetic organization and the origin of unrelated workers in a eusocial sweat bee, *Lasioglossum malachurum*

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Abstract. Sweat bees (Halictidae) exhibit great interspecific and intraspecific diversity in their social organisation, yet there is remarkably little information on the sociogenetic organisation of any species. *Lasioglossum malachurum* is a eusocial sweat bee with an annual lifecycle that exhibits considerable variation in its social organisation across its wide geographic range from northern to southern Europe. We collected all adults from 31 *L. malachurum* nests at Eichkogel, Austria, near the latitudinal centre of its distribution, and genotyped 148 workers using 5 highly variable microsatellite loci developed for this species. Nests were often queenless (48% of nests) during the second phase of worker activity, when colonies were provisioning the sexual brood. Pedigree reconstruction and estimates of nestmate genetic relatedness demonstrated that nests often (32% of nests) contained alien workers, probably as a result of worker drifting from their natal to a foreign nest. Queen effective mating frequency was variable (harmonic mean $m_e = 1.24$), but sometimes high (maximum 2.7). These data demonstrate that nests of *L. malachurum* do not have a classical eusocial sociogenetic organisation (monogyny, monandry) and thereby pose a challenge to exclusively relatedness based arguments for the evolution of eusociality in the taxon.

Keywords: Eusociality, microsatellites, relatedness, foreign workers, Halictidae.

Introduction

Sweat bees exhibit great intraspecific and interspecific diversity in their social behaviour. Some species are solitary, others communal or obligately eusocial, some are facultatively eusocial (Sakagami and Munakata, 1972; Packer, 1990; Soucy, 2002; Hirata and Higashi, 2008), and the group includes at least one species, *Lasioglossum marginatum*, with a perennial colony cycle (Plateaux-Quénu, 1959). One of the reasons for the social diversity and intraspecific flexibility of halictid bees may be their relatively recent origin of eusociality (approximately 20–22 mya), much later than in other major groups of eusocial Hymenoptera: ants, vespid wasps and apid bees (Brady et al., 2006). Given their broad social diversity, halictid bees are good models to study social evolution (Schwarz et al., 2007), particularly questions related to the origin of sociality (Mueller, 1991; Mueller et al., 1994; Soucy and Danforth, 2002). Yet there is remarkably little information on their sociogenetic organization with which to parameterise models of social evolution (cf. Kukuk, 1989; Richards et al., 1995, 2005; Paxton et al., 2002) or with which to test current hypotheses for the origin of sociality (cf. Mueller, 1991; Mueller et al., 1994).

A major question in social evolution concerns the ecological factors and evolutionary forces responsible for the origin and maintenance of social diversity, which may be addressed by comparing intraspecific diversity in colony sociogenetic organization at different locations within the distributional range of a species. *Lasioglossum malachurum* is one of the best studied eusocial halictid bees, common and widespread across Europe, that exhibits considerable geographic variation in colony social organization (Knerer, 1992; Richards, 2000; Wyman and Richards, 2003). Like other temperate

eusocial sweat bees, *L. malachurum* has a typical colony cycle comprising spring colony establishment and provisioning of the first worker brood by an overwintering foundress, additional discrete phases of worker brood provisioning and a terminal phase of sexual brood development (Knerer, 1992; Schwarz et al., 2007). In cooler climates *L. malachurum* produces only a single worker brood before the reproductive brood, while in warmer locations with longer breeding seasons it produces up to three worker broods before the gyne and male brood, though a small proportion of males may also be produced within worker broods (Packer and Knerer, 1985; Knerer, 1992).

It has been hypothesised that the expression of eusociality in sweat bees, as measured by queen-worker reproductive skew, increases under harsher environmental conditions or in areas with a shorter breeding season (Packer and Knerer, 1985; Richards, 2000). This pattern seems to occur because harsher conditions require increased worker cooperation in nest maintenance and brood production. A corollary is that there is reduced reproductive skew (i.e. greater worker reproduction) with longer seasons. Knerer (1992), Richards (2000) and Wyman and Richards (2003) have studied geographic variation in the social organization of *L. malachurum* across Europe from morphological, physiological and demographic points of views. In agreement with the hypothesis of increased expression of eusociality under harsher conditions, *L. malachurum* workers have been reported to exhibit lower rates of ovarian development in northern versus southern populations (Packer and Knerer, 1985; Knerer, 1992; Richards, 2000; Wyman and Richards, 2003; cf. Strohm and Bordon-Hauser, 2003).

Yet despite *L. malachurum* having been the subject of extensive investigation, there are only two studies of its sociogenetic organization in which genetic markers have been used to characterise the relatedness structure of colonies and accurately determine reproductive skew. Paxton et al. (2002) studied a population in Tübingen (Germany) which, with only one worker brood, exemplifies a northern type of colony cycle (i.e. a harsh environment). Richards et al. (2005) studied an aggregation of *L. malachurum* in Agios Nikolaos Monemvasias (ANM) (southern Greece) which, with three worker broods, represents a southern type of colony cycle (i.e. a non-harsh or mild environment). At both localities, queen mating frequency was low and colonies were either strictly monogynous (Tübingen) or exhibited a low frequency of polygyny (2 queens per nest in ANM). However, in contrast to the hypothesis that harsh environments select for great skew, Paxton et al. (2002) found considerable worker reproduction (19% of gyne brood) in Tübingen whilst Richards et al. (2005) found little worker reproduction (2–4% of sexuals) in ANM, despite the majority of workers showing some degree of ovarian development (Wyman and Richards, 2003).

The notable contrast between these two sociogenetic studies is that nests in Tübingen frequently (33% of nests)

contained foreign or alien workers unrelated to the queen, and it was these alien workers that reproduced in the presence of the queen, supporting concessions models of reproductive skew (Paxton et al., 2002). Southern Greek colonies contained alien workers less frequently (17% of nests) and there was no evidence of their having reproduced (Richards et al., 2005). Paxton et al. (2002) hypothesised colony usurpation by unrelated queens before worker emergence as the source of the alien workers i.e. workers in their natal nest became aliens due to the replacement of their own queen by an unrelated, usurping queen during the colony foundress phase (Zobel and Paxton, 2007), a phenomenon that may occur in other eusocial sweat bees (e.g. *Lasioglossum zephyrum*, Kukuk et al., 1987). Knerer (1992) also notes that colony usurpation by *L. malachurum* foundresses occurs prior to worker emergence but that queens are not seen above ground following worker emergence; colony usurpation by queens therefore cannot occur following worker emergence. This could explain the rarity of alien workers in ANM populations of *L. malachurum*; with three phases of worker brood production in southern Greece, aliens arising due to usurpation are unlikely to survive through to the colony reproductive phase. Another source of alien workers is via 'drifting', the active or passive movement of workers from one nest to another (see Pfeiffer and Crailsheim, 1998). Interestingly, drifting workers have been suggested to contribute disproportionately to colony reproduction, both in annual eusocial bumble bees (Lopez-Vaamonde et al., 2004) and perennial eusocial honeybees when colonies become queenless (Nanork et al., 2005, 2007). For *L. malachurum*, both colony usurpation by queens and worker drifting remain viable routes by which workers become alien.

To understand better the degree of variation in the sociogenetic organisation of *L. malachurum* across its range and to determine the origin of alien workers, we used microsatellites to describe the relatedness structure of colonies at Eichkogel (Austria), where the population has two worker broods and is therefore intermediate between Tübingen and ANM in its colony demography. We examined the second worker brood pedigree structure and deduced from it the queen's mating frequency and mate relatedness as well as the presence and putative origin of alien workers.

Materials and methods

Social biology of the study species

Lasioglossum malachurum is a primitively eusocial sweat bee common throughout the western Palearctic and has been extensively studied in central and southern Europe, where it is a common and widespread ground-nesting bee (reviewed in Michener, 1974; Westrich, 1989; Knerer, 1992). Nesting aggregations with up to several thousand nests can be found in bare or sparsely vegetated soil. Seasonal activity starts in early spring (March–April) when colonies are founded by single overwintered gynes (cf. Richards et al., 2005). The first brood consists

primarily of workers although, depending on climatic region, a few males can occasionally be found (Packer and Knerer, 1985; Knerer, 1992). From the last brood of the year (the third brood in Eichkoggl), first males and several days later female reproductives emerge. The species is always eusocial and workers are not thought to found their own nests (cf. Wyman and Richards, 2003; Richards et al., 2005), whilst the queen never departs her nest after the solitary foundress phase. Given its broad geographic distribution across Europe, the Mediterranean Basin and to the Caucasus, much variation in colony demography of *L. malachurum* has been described (Knerer, 1992), and populations in warmer locations may have two (as in Eichkoggl) or three primarily worker broods before the colony produces its final all-sexual brood, by which time the queen is often already dead (Richards, 2000; Wyman and Richards, 2003). This annual colony cycle is typical of many primitively eusocial halictids in temperate climates (Schwarz et al., 2007).

Bee collection

Bees were collected at the aggregation Eichkoggl (near Vienna, Austria) at the end of July and beginning of August 1995 (Table 1), when second brood workers were provisioning the final, purely sexual brood, frozen till dissection and thence stored in 99% ethanol till DNA extraction. Bees were taken from the nest entrance using a vacuum suction device (a large pooter with clear plastic suction tube) after slightly enlarging the entrance with a spatula. By carefully sucking up each individual as it appeared at the nest entrance, all workers in the nest and the queen were collected without having to excavate the nest. When a queen was present, it was always the last and largest individual from a nest to be collected with well worn wings (queens are 15% larger than workers, see Packer and Knerer, 1985). Sampled nests were devoid of bees on days following collection, suggesting that most, if not all individuals of a nest were collected using this methodology. As some second brood workers may have died before sampling, we cannot estimate the total worker complement of the second brood. In addition some collected workers may have survived from the first brood.

Microsatellite analysis

DNA was extracted from the thoraces of 148 worker bees collected at the entrances of 31 nests using a high-salt protocol (Paxton et al., 1996) and all individuals were analysed at five microsatellite loci developed specifically for this species (see Paxton et al., 2003 for loci mala01, mala04, mala07 and mala09). Locus mala11 is new and has PCR primer sequences (3'->5') Forward: GAAGGACAGGCGA-TAAAGC, Reverse: CTTTACTGCGGAGTAATGGTATT (GenBank Accession No. BV728880); it was developed with the other, published loci (Paxton et al., 2003). PCR amplifications were performed in 10 µL reaction volumes using an MJ Research PTC-100 thermal cycler. Individual mixes consisted of 1 µL template DNA (ca. 10 ng), 4 pmol of each primer, dGTP, dCTP and dTTP at a final concentration of 75 µM each, dATP at a final concentration of 6 µM, 0.125 µcurie [$\alpha^{33}\text{P}$]-dATP, 0.4 units of Taq DNA polymerase (Promega) and 1 µL of DNA polymerase 10x Buffer (Buffer B, Promega) at a final MgCl_2 concentration of 1.5 mM. Samples went through one denaturing step of 3 min at 94 °C, followed by 28 cycles consisting of 30 s at 94 °C, 30 s at the annealing temperature specified in Table 2, and 45 s at 72 °C, with a final elongation step of 10 min at 72 °C. Loci mala04 and mala09 were amplified in one PCR mix, and loci mala01 and mala11 were amplified in a second multiplex. For the latter PCR, the denaturing step was prolonged to 45 s, the elongation step was prolonged to 1 min 30 s, and the number of cycles was increased to 35. Changes were successfully introduced in order to avoid long allele dropout (Schlötterer, 1998), hypothesized on previous results. Alleles were resolved in 43 cm long 'sequencing' gels (8M Urea, 6% polyacrylamide) and visualised by autoradiography. In every gel and for all loci, two individuals of known genotype were run simultaneously with test individuals. All gels were independently scored twice and, whenever ambiguities in allele calling arose, individuals were reamplified and visualised at that locus.

Table 1. Contents of 31 *Lasioglossum malachurum* nests and effective mating frequency of the queen (m_e)^a

Nest	Collection date	Queen present	<i>n</i> workers present		
			queen's	Aliens	<i>n</i> patrines (m_e)
1	25/07/1995	yes	2		1
2	25/07/1995	no	3	1	2 (1.8)
3	25/07/1995	yes	2		1
4	25/07/1995	yes	5		2 (1.5)
5	25/07/1995	yes	4		1
6	25/07/1995	no	1		–
7	25/07/1995	no	4		1
8	25/07/1995	no	1		–
9	25/07/1995	no	1		–
10	25/07/1995	no	3		–
11	27/07/1995	no	3	1	2 (1.8)
12	27/07/1995	yes	7		2 (2.0)
13	27/07/1995	no	9		1
14	27/07/1995	no	3		1
15	27/07/1995	no	4		1
16	31/07/1995	yes	9		2 (1.8)
17	31/07/1995	yes	7	1	1
18	31/07/1995	yes	6	1	1
19	31/07/1995	yes	4	1	1
20	02/08/1995	yes	8		1
21	02/08/1995	yes	7	1	2 (1.7)
22	02/08/1995	yes	8		2 (1.3)
23	02/08/1995	yes	7		1
24	02/08/1995	yes	8		1
25	02/08/1995	yes	4		2 (1.6)
26	02/08/1995	yes	3		1
27	27/07/1995	no	1		–
28	27/07/1995	no	1		–
29	31/07/1995	no	4	1	3 (2.7)
30	31/07/1995	no	7		3 (2.3)
31	31/07/1995	no	4	1	2 (2.0)
		16 yes	Total	Total	Mean
		15 no	140	8	1.52 (1.24 ^b)

^a Excludes alien offspring

^b Harmonic mean

Genetic marker variability

Observed heterozygosity (H_{obs}) at the five microsatellite loci, Nei's (1978) unbiased estimate of heterozygosity (H_{exp}), and the inbreeding coefficient, F_{IS} [as $(H_{\text{exp}} - H_{\text{obs}})/H_{\text{exp}}$] were calculated by hand from the genotypes of a subsample of 31 workers (one per nest). The same subsample was used to evaluate genotypic disequilibrium among loci and the significance of deviation from Hardy-Weinberg equilibrium using GENEPOP 4.0 (Raymond and Rousset, 1995).

Table 2. Variability of the microsatellite loci in *Lasioglossum malachurum* at Eichkogel and the annealing temperature of the PCR (T_m). Number of unique alleles is based on the entire data set. Observed heterozygosity (H_{obs}), expected heterozygosity (H_{exp}) and coefficient of inbreeding (F_{IS}) are calculated from the genotypes of 31 workers (one per nest). Mutation rates (mutations per meiosis) are inferred from nests with more than one worker.

Locus	n unique alleles	T_m (°C)	H_{obs}	H_{exp}	F_{IS}	Mutation rate
mala01	18	55	0.935	0.925	-0.011	0
mala04	25	60	1.000	0.977	-0.024	0.010
mala07	13	60	0.774	0.791	+0.059	0.003
mala09	7	60	1.000	0.816	-0.225	0.006
mala11	21	55	0.903	0.909	+0.006	0

Sociogenetic organization

Queens were not genotyped as they were used for odour analysis and were not available for DNA extraction. Therefore, to determine a colony's pedigree structure, we inferred queen and fathering male genotypes on the basis of workers (see Estoup et al., 1994 for the logic). With few workers per nest, MateSoft (Moilanen et al., 2004) was unsuitable as a software to support pedigree reconstructions. For 5 of 31 nests, only one worker was collected (Table 1) and it was therefore not possible to infer the genotypes of the putative parents. For a sixth nest (nest 10), genotypes of the three workers also did not allow straightforward assignment of maternal and paternal alleles, leaving 25 nests from which we could unambiguously infer colony pedigree structure.

The population-wide probability of genetic non-detection of a second fathering male among the offspring of a nest (d_p) was calculated as the probability that two males had identical genotype at all investigated loci using:

$$d_p = \prod(\sum q_i^2) \text{ (Boomsma and Ratnieks, 1996)}$$

where q_i is the frequency of the i th allele, summation is over alleles at a locus and multiplication is over loci. The probability of genetic non-detection of an additional matriline amongst a set of putative daughters (d_m) was calculated at the colony level as the sum of the frequencies of the queen alleles at each heterozygous locus or the frequency of the queen allele at each homozygous locus (q_i), multiplied across loci, namely:

$$d_m = \prod(\sum q_i) \text{ (Palmer et al., 2002)}$$

where q_i is the frequency of the i th queen allele, summation is over a queen's alleles at a locus and multiplication is over loci.

When the contributions of fathers to a single matriline's daughters in a colony were unequal, we calculated the effective mating frequency (m_e), defined as a harmonic mean:

$$m_e = 1/(\sum y_i^2) \text{ (Starr, 1984)}$$

where y_i is the proportion of the daughters fathered by the i th male and summation is over fathering males. A point estimate of the relatedness between daughters within a colony (pedigree relatedness, g_D) was then computed as:

$$g_D = [0.75 + (m_e - 1)g_p] / m_e \text{ (Crozier and Pamilo, 1996)}$$

where g_p is the relatedness between daughters in different patrilines, assumed to be 0.25, which is the case if the fathering males are unrelated. In eight colonies a worker was found which lacked a queen allele at three or more loci and therefore could not have been a daughter of the nest's putative queen, the mother of the other workers. For these nests, a point estimate of the mean pedigree relatedness for daughter nestmates of both matriline was calculated as:

$$g_{D+A} = p_D^2 * g_D + p_A^2 * g_A \text{ (Paxton et al., 2001)}$$

where p_D and p_A are, respectively, the proportion of the queen's own daughters and those of the other matriline (aliens), g_A is the relatedness among aliens, and the matriline is considered to be unrelated.

The algorithm of Queller and Goodnight (1989), as implemented in RELATEDNESS 5.0.8, was additionally used to obtain estimates of genetic (regression) relatedness, b , among nestmate workers and among a colony's putative fathering male haplotypes. Colonies were weighted equally and allele frequencies were calculated by excluding alleles of a focal nest. Under the assumptions that multiple matriline and the multiple fathers of a single matriline within a colony are unrelated, pedigree relatedness (g_D or, for nests with aliens, g_{D+A}) and genetic relatedness (b) estimates of nestmates are expected to be identical. Standard errors of b were obtained by jack-knifing across loci.

Dissections and measurements

Of the 148 workers that were genotyped, 125 were also dissected to determine the size of their fat bodies, evaluated with a nominal scale from 1 (undeveloped) to 3 (well developed), and the developmental stage of their ovaries, with a scale from 1 (undeveloped) to 5 (oocyte fully developed and ready for laying) (see Michener, 1974). Though *L. malachurum* workers can mate and though not only the final brood but also the preceding phases contain some males (Knerer, 1992), males were not seen and spermathecae were not examined for the presence of sperm. Head width was also measured under a dissection microscope with an eyepiece graticule (± 0.05 mm) and wing wear was evaluated on a nominal scale from 1 (unworn) to 4 (wings heavily notched) (see Michener, 1974). Queen heads were destroyed for odour extraction and therefore were not available for measurement.

Differences among groups of workers were evaluated using non-parametric (fat bodies, ovaries, wing wear) and parametric (head widths) statistics, treating each worker as an independent unit. As there may be non-independence of workers from the same nest, this may inflate degrees of freedom, an issue we highlight below. Means are presented throughout \pm SE.

Results

Microsatellite variability

The five microsatellite loci showed high variability, as indicated by the large number of unique alleles per locus and by high observed and expected heterozygosities (Table 2). Loci were not in linkage disequilibrium ($P > 0.05$ for all pairwise comparisons) and did not exhibit significant deviation from Hardy-Weinberg equilibrium

($P > 0.05$ for all loci). Due to the high variability of the five loci, the population-wide probability of non-detection of a second male's offspring due to two males having identical genotypes, d_p , was very small ($P < 0.00002$). The probability of genetic non-detection of a second matriline among a colony's workers, d_m , was similarly low (P from 0.002–0.000003). The markers therefore had excellent resolving power for determining the genetic structure of nests and identifying additional patrilines or matriline.

In each of six colonies (nests 7, 12, 16, 22, 30 and 31) one individual was found which lacked either a paternal or a maternal allele at only one of the five loci. All such individuals were re-amplified two more times but allele calling remained the same. In these cases, instead of implying a further patriline or matriline, we assumed that these non-parental alleles were the result of a mutation. Changes in allele length were by a single repeat motif, a 'one step' mutation, in 5 cases and a 'two step' mutation in the remaining case. Mutation rates per locus were calculated from nests with more than one worker, and ranged from zero to 0.01 (Table 2). All subsequent results are based on the assumption that these six non-parental alleles were products of mutational events. All other exceptions from strict monandry and monogyny were due to individuals lacking a paternal or a maternal allele at four or all five loci. Ignoring putative mutational events would lead to slight inflation of mating frequencies reported below.

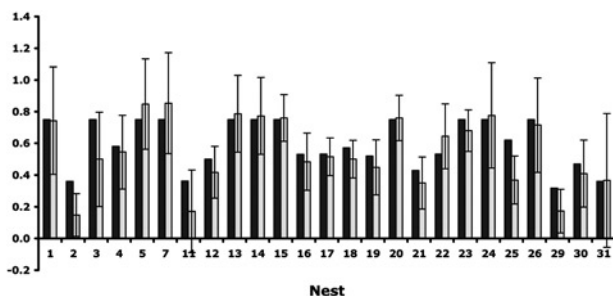


Figure 1. Relatedness among *LasioGLOSSUM malachurum* nestmates (workers) from 25 nests (see Table 1 for sample sizes). In black: point estimate of pedigree relatedness, g_D or g_{D+A} for nests with aliens; in white: b , genetic relatedness (Queller & Goodnight, 1989) with 95% CIs. Queen's workers and aliens included in both g_D/g_{D+A} and b .

Sociogenetic organization

Colonies only ever contained a single queen (monogyny). Yet in 15 of the 31 nests the queen was not found (Table 1). Though queenless nests contained fewer workers (3.6 ± 0.6) compared to queen-right nests (5.1 ± 0.6), the difference was not significant ($t_{29} = -1.713$; $P = 0.097$).

In 32% ($n = 8$) of the 25 nests used for pedigree analysis, unrelated (alien) workers of a second matriline were found, one alien in each of 4 queen-right and 4 queenless nests (Table 1). Queenless nests were not more

or less likely to contain alien workers than queen-right nests (Fisher exact test, $P = 0.689$). Alleles of aliens differed from those of the inferred queen's workers (i.e. differed from the major matriline and patrilines) and from the inferred queen's alleles at 4–5 loci. In addition, genetic relatedness between a queen's own workers and alien nestmates was not significantly different from zero ($b = -0.019 \pm 0.062$; $t_7 = -0.509$; $P = 0.626$). In three cases (alien workers in nests 11, 18 and 19), alien genotypes were consistent with those of workers resident in other nests (nests 15, 19 and 18 respectively; genetic relatedness of these three aliens to their putative natal nestmates: $b = 0.703 \pm 0.038$), a good indication that they had drifted into a foreign nest.

Excluding aliens, 38 patrilines and 25 matriline could be inferred among the remaining 130 worker genotypes of the 25 nests used for pedigree analysis (Table 1). Based on worker genotypes, pedigree reconstruction of the 25 nests revealed 56% of colonies ($n = 14$ nests) with a single patriline, 36% of colonies ($n = 9$ nests) with two patrilines and three patrilines were found in two colonies (8%) (Table 1). For those colonies with two or more patrilines, effective mating frequency (m_e) varied between 1.3 and 2.7 (see Table 1).

Nestmate genetic relatedness, b , was 0.612 ± 0.020 , calculated excluding alien workers ($n = 130$ workers), and 0.560 ± 0.020 calculated including alien workers ($n = 138$ workers). Though our estimates of pedigree relatedness largely agreed with estimates of genetic regression relatedness (see Fig. 1), there was a marginally significant difference between the two (paired t-test $t_{24} = 2.097$; $P = 0.047$). Pedigree relatedness (mean across nests = 0.597 ± 0.031) was slightly higher than b , and significantly so for three nests (2, 25 and 29; see Fig. 1). We think that this subtle difference is due to the failure of b to identify full sisters, rather than violations of the assumption of matriline and patriline within a colony being unrelated. Indeed, matriline were unrelated (see above) and relatedness between inferred fathering haplotypes in nests with multiple patrilines ($n = 11$ nests) was not significantly different from zero ($b = 0.043 \pm 0.039$; $t_{10} = 0.803$; $P = 0.441$).

Dissections and measurements

Dissection of 125 workers revealed that most had abundant fat bodies (mean score = 2.568 ± 0.069 ; range: 1 – 3) and that their ovaries were at variable, occasionally advanced stages of development (mean score = 1.660 ± 0.061 ; range: 1 – 4, but only one individual scored 4). The mean head width of workers was 2.097 ± 0.007 mm, within the usual range of values shown by workers of *L. malachurum* at other locations (Richards, 2000).

Five of the 8 identified aliens were among the 125 measured workers. These aliens did not differ in fat body status from the 24 non-aliens found in the same nests

(Mann-Whitney U-test $n = 5$ and 24 for aliens and non-aliens respectively; mean scores = 2.400 and 2.792 respectively; $Z = 0.924$; $P = 0.356$), they did not differ in their ovary status ($n = 5$ and 24 for aliens and non-aliens respectively; mean scores = 2.000 and 1.708 respectively; $Z = 0.173$; $P = 0.863$) and they did not differ in size from non-aliens ($n = 5$ and 24 for aliens and non-aliens respectively; mean head width = 2.080 mm and 2.067 mm respectively; $Z = 0.465$; $P = 0.644$). Also the wing wear of aliens did not differ significantly from non-aliens ($n = 1$ and 24 for aliens and non-aliens respectively; mean scores = 1.800 and 1.667 respectively; $Z = 0.736$; $P = 0.525$). Results obviously remain non-significant when accounting for the statistical non-independence of workers from the same nest. Thus, within nests that contained them, aliens were not the most extreme in fat body status, ovary development, size or wing wear.

Surprisingly, workers retrieved from nests without a queen ($n = 11$ nests) did not differ from those retrieved from nests with a queen ($n = 16$ nests) in fat body status (Mann-Whitney U-test $n = 41$ and 84 for queenless and queen-right workers respectively; mean scores = 2.463 and 2.619 respectively; $Z = 1.085$; $P = 0.278$) or ovarian development (Mann-Whitney U-test $n = 41$ and 84 for queenless and queen-right workers respectively; mean scores = 1.707 and 1.637 respectively; range $1-3$ and $1-4$ respectively; $Z = 0.718$; $P = 0.473$). Queenless workers did, however, exhibit slightly reduced wing wear compared to queen-right workers (Mann-Whitney U-test $n = 41$ and 84 for queenless and queen-right workers respectively; mean scores = 1.171 and 1.452 respectively; $Z = 2.545$; $P = 0.011$), a difference that remains significant when accounting for the non-independence of workers from the same nests.

Discussion

There are three primary results that arise from our analyses. Colonies were often queenless at a time when the sexual brood was being produced. Alien workers were often detected, and probably arose through drifting from neighbouring nests. Colonies were monogynous yet queen mating frequency was variable and sometimes high ($m_e > 2$).

Matricide

At Eichkogel 48% of colonies were without a queen during the sexual brood provisioning phase, though these nests did not differ from queen-right nests in terms of worker complement. A caveat is that our method of adult collection may mean we slightly overestimate queen loss. Nevertheless, moderate rates of queen loss (17%) have been reported in Greek populations of *L. malachurum*, also during the final provisioning phase when the sexual

brood was being produced (Wyman and Richards, 2003; Richards et al., 2005). Kin selection theory predicts worker matricide of the queen towards the end of the annual colony cycle in primitively eusocial Hymenoptera, and observational data support this contention in primitively eusocial bumble bees and vespid wasps (review in Bourke, 1994). Though it is not possible to determine through direct observation the cause of queen loss in fossorial species like *L. malachurum*, it is nevertheless clear that the absence of queens in many *L. malachurum* nests may release workers from queen social control or reproductive suppression and therefore give them the opportunity to lay sexual-destined eggs. That queenless workers had less wing wear than queen-right workers suggests that they foraged less and spent more time in the nest. However, queenless workers were no different in ovarian status and fat body status to queen-right workers. This may suggest that both are potentially reproductively active (see also Strohm and Border-Hauser, 2003). Direct reproduction by *L. malachurum* workers may be more widespread than has hitherto been considered the case (e.g. Packer and Knerer, 1985; Knerer, 1992; Paxton et al., 2002), but cannot be verified in our study because data on worker spermathecal content and of the genotypic profile of reproductive offspring are lacking.

Alien workers

Alien workers were found in approximately a quarter of the nests at Eichkogel. Alien workers were probably drifters from other nests in the aggregation, rather than daughters of a second queen (polygyny) or daughters of a usurped queen (serial monogyny). Firstly, they were always found as singletons and, secondly, in three cases their genotypes matched them to other nests that had been sampled.

Alien workers have been detected, or inferred to occur, at moderate frequencies in other populations of *L. malachurum*. In 33% of *L. malachurum* colonies in Tübingen, alien workers or gyne pupae derived from aliens were detected, though it was suggested that aliens were the offspring of former, usurped queens rather than drifters (Paxton et al., 2002; see also Kukuk et al., 1987). Richards et al. (2005) detected 11 alien workers in 9 of 52 (17.3%) Greek colonies; aliens likely arose through drifting. The cumulating evidence suggests that alien workers are a regular feature of the sociogenetic structure in *L. malachurum* colonies.

From a proximate perspective, there is good evidence for odour based individual and nest recognition mechanisms in *L. malachurum* (Ayasse, 1990 and references therein), and therefore it is surprising that we infer moderate levels of worker drifting, even if occasional nest recognition errors cannot be excluded. From an ultimate perspective, the benefit to the drifter of drifting may be enhanced chances of direct reproduction. Paxton et al. (2002) demonstrated that alien *L. malachurum* workers,

but not own workers, reproduced in the presence of the queen. In an analogous case of intraspecific social parasitism, drifting workers of *Bombus terrestris* produced significantly more males per capita than resident workers (Lopez-Vaamonde et al., 2004). More recently, drifting workers have been shown to have enhanced reproduction in eusocial honeybees when colonies become hopelessly queenless and workers commence laying male-destined eggs (Nanork et al., 2005, 2007). Workers of *L. malachurum* may therefore drift to another colony to enhance their direct reproduction, though our data on their ovarian status were equivocal; aliens appeared to be no different to other nestmates in ovarian development. Genetic analysis of reproductive offspring is needed to support the hypothesis of drifting to enhance direct reproduction in this halictid bee. The question of why drifting workers are accepted into a foreign nest remains open, though shared use of the same resource (Sorvari et al., 2008) and occasional recognition errors may provide a proximate explanation.

Monogyny and polyandry

Colonies of *L. malachurum* at Eichkogel were strictly monogynous, though they may have contained alien workers. They therefore resemble those at Tübingen, where only monogyny was detected (Paxton et al., 2002). Reports of polygyny in *L. malachurum* are currently confined to one population in Greece (Wyman and Richards, 2003; Richards et al., 2005), the south of the range of the species, and may reflect the large worker complement that colonies attain with three phases of worker brood production, rather than two phases (Eichkogel) or one (Tübingen) of other genotyped populations.

Queen mating frequency of *L. malachurum* at Eichkogel was variable (1–3), but sometimes high (m_e maximum 2.7, harmonic mean 1.24). Again, these data are consistent with those of Tübingen (mating frequency 1–4, m_e maximum 3.0, harmonic mean 1.29; Paxton et al., 2002). Multiple mating was less frequent in southern Greece (Richards et al., 2005), where only 3% of queens mated twice. Whether m_e in *L. malachurum* is inversely related to queen number at the population level is unclear as it is confounded by colony size (worker complement), that is much higher in southern than northern populations due to the longer colony cycle and increased number of worker brood phases that a long season permits.

Despite the possible advantages of multiple mating as a source of genetic diversity (Tregenza and Wedell, 2002; Oldroyd and Fewell, 2007; Hughes et al., 2008b), its general rarity among social Hymenoptera (Boomsma and Ratnieks, 1996; Strassmann, 2001) is expected if it is assumed that strict lifetime monogamy is the single pervasive condition for the evolution of eusociality (Hamilton, 1964; Boomsma, 2007; Hughes et al., 2008a). Multiple mating in social Hymenoptera can be explained within a relatedness based conceptual frame-

work as a secondary development, after workers had irreversibly lost the capacity to mate (Hamilton, 1964, 1972; Wilson, 1971; Andersson, 1984; Boomsma, 2007). Given the three relatively recent origins of eusociality in the Halictidae, and that *L. malachurum* is obligately eusocial though workers can mate and lay haploid and diploid eggs (Knerer, 1992; Paxton et al., 2002), our observation of polyandry in *L. malachurum* suggests the taxon may represent a challenging test case for the solely relatedness based hypothesis that monandry permitted the evolution of eusociality in the Hymenoptera (Boomsma, 2007), shifting emphasis from the relatedness term to the benefits and costs terms of Hamilton's rule. Indeed, the moderate rates of ovarian development of workers at Eichkogel suggest that workers reproduce in the presence of the queen. Queens were unable completely to suppress ovarian development when there were as few as four workers in a Würzburg (Germany) population of *L. malachurum* (Strohm and Bordon-Hauser, 2003). Thus, despite the small worker complement per colony, *L. malachurum* seems to lack queen control or coercion of workers (Ratnieks and Wenseleers, 2008), in both northern (Tübingen) and central (Eichkogel) populations.

Microsatellite analyses

The microsatellites we used had excellent resolving power to detect matriline and patriline among nestmates due to their high heterozygosity. However, their high rates of mutation (Ellegren, 2004) may cause difficulty in pedigree reconstruction, as we observed in our data set, warranting the use of several loci and caution in data interpretation. Even with five polymorphic microsatellite loci, we found estimates of nestmate genetic relatedness (Queller and Goodnight, 1989) to underestimate those from pedigree reconstruction in three of 25 cases, suggesting that more than five loci would be needed for greater accuracy of *b*.

The use of high resolution molecular genetic markers has nevertheless made it possible to detect unrelated individuals among nestmates in *L. malachurum* and in other primitively eusocial Hymenoptera, too (e.g. Queller et al., 2000; Lopez-Vaamonde et al., 2004; Zhanette and Field, 2008). Recently, Sumner et al. (2007) have recorded individual *Polistes canadensis* wasps fitted with radio-transmitters to engage in considerable inter-nest movement. Combining observational with genetic methods would allow greater insight into the behavioural flexibility of individuals as well as the consequences of their actions.

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