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Do hornets have zombie workers?

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Abstract

Colonies of the European hornet, *Vespa crabro*, are typically founded by a single queen mated to a single male. From the resulting colony relatedness pattern we predicted strong worker–queen conflict over male production where both the workers and the queen attempt to produce the colony's males. To test for this conflict, male production was studied in 15 hornet nests using a combination of DNA microsatellite analysis (282 males), worker ovary dissections (500 workers from eight nests) and 50 h of observation (four nests). In contrast to our prediction, the data show that hornet males are queens' sons, that workers never attempt to lay eggs, rarely have activated ovaries, and that there is no direct aggression between the queen and the workers. This contrasts with other data for vespine wasps, which support relatedness predictions. *Dolichovespula arenaria* has the same kin structure as *V. crabro* and workers produce males in many colonies. The similarity between these two species makes it difficult to explain why workers do not reproduce in *V. crabro*. Self-restraint is expected if worker reproduction significantly reduces colony productivity but there is no obvious reason why this should be important to *V. crabro* but not to *D. arenaria*. Alternatively, queen control may be important. The absence of expressed queen–worker conflict rules out physical control. Indirect pheromonal control is a possibility and is supported by the occurrence of royal courts and queen pheromone in *Vespa* but not *Dolichovespula*. Pheromonal queen control is considered evolutionarily unstable, but could result from a queen–worker arms race over reproductive control in which the queen is ahead. The genetic data also revealed diploid males in one colony, the first example in the vespine wasps, and two colonies with double matrilines, suggesting that occasional usurpation by spring queens occurs.

Keywords: conflict, diploid males, DNA microsatellites, male production, queen pheromone, *Vespa crabro*

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Introduction

Insect societies are often considered superorganisms composed of cooperating individuals (e.g. Wheeler 1911; Wilson 1985; Wilson & Sober 1989). While cooperation is fundamental, there is also the potential for conflict (Ratnieks & Reeve 1992). The eusocial Hymenoptera are particularly interesting in this respect owing to their diverse kin structures and haplodiploid genetics, which cause great diversity in potential intracolony conflicts. One major area of potential conflict is male production. Being haploid, males can be offspring of both unmated workers and queens. In a colony headed by a singly mated queen, workers should prefer rearing sons ($r = 0.5$) and other workers' sons ($r = 0.375$) to their mother's sons ($r = 0.25$) (Hamilton 1964; Trivers & Hare 1976; Ratnieks 1988). Relatedness, therefore, predicts that workers will conflict with the queen over male production, both individually and collectively.

Actual conflict over male production may be reduced in colonies with effective paternities (queen mating frequency) above two (Starr 1984; Woyciechowki & Lomnicki 1987; Ratnieks 1988), or many closely related queens (Pamilo 1991), because workers are then more related to the queens' sons than other workers' sons. Although individuals are always most related to their own sons, the workers' collective interests are now aligned with the queens' interests. Increased cooperation can then occur through collective worker policing of individual workers

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resulting in queen-only male production (Ratnieks 1988). However, with low paternity common in Hymenoptera (Boomsma & Ratnieks 1996) and workers being the numerically dominant power, (Trivers & Hare 1976; Keller & Nonacs 1993) actual queen–worker conflict over male production is expected to be widespread.

Worker reproduction in colonies with a queen has been recorded several times in the eusocial Hymenoptera but its occurrence is highly variable between and within species (Bourke 1988; Bourke & Franks 1995). Assessing the role of colony kin structure was initially difficult due to the requirement for accurate data on paternity or queen relatedness (Keller & Vargo 1993). The first such data came from visible genetic markers (e.g. Owen & Plowright 1982) and allozymes (e.g. Pamilo 1982). These are now supplemented by more powerful DNA microsatellite studies (Queller *et al*. 1993). The potential power of relatedness predictions has been demonstrated by comparing stingless bees (Meliponinae) with the honeybee *Apis mellifera* (Ratnieks 1988; Peters *et al*. 1999). *Apis* has extremely high paternity (Estoup *et al*. 1994) and workers that police each other's reproduction (Ratnieks & Visscher 1989). This contrasts with low paternity in stingless bees (13 species, Peters *et al*. 1999), ritualized queen–worker conflict (Kerr 1969) and worker laying (Sakagami 1982). In further agreement, low paternity in bumblebees (5 species, Estoup *et al*. 1995; Thorén 1998) is associated with intracolony conflict and worker male production (Honk *et al*. 1981).

Data from the vespine wasps are consistent with relatedness predictions. Two *Vespula* species have high paternities and evidence of queen only male production (*V. maculata and V. squamosa* Ross 1986). This contrasts with its sister group *Dolichovespula* where effective paternities below two have been found in six species (*D. arenaria*, F. L. W. Ratnieks and J. J. Boomsma, unpublished; *D. maculata*, *D. sylvestris*, *D. norwegica*, *D. media* and *D. saxonica*, K. R. Foster and F. L. W. Ratnieks, unpublished) and worker male production occurs in queenright colonies (*D. arenaria*, Greene *et al*. 1976; F. L. W. Ratnieks and J. J. Boomsma, unpublished).

Not all data, however, agree with relatedness predictions. Paternity below two but no worker reproduction was reported in 14 colonies of *Bombus hypnorum* (Thorén 1998). Walin *et al*. (1998) analysed three *Formica* and one *Myrmica* ant species and showed that while relatedness predicted worker male production in all, it could only be considered a possibility in one. In addition, patterns of male production in *Leptothorax* ants do not seem to be attributable to kin structure alone (Heinze *et al*. 1997). These studies demonstrate that relatedness is not the only factor affecting worker reproduction in queenright colonies. Costs of worker reproduction on colony productivity and queen control may also be important, although their precise role remains uncertain due to the difficulties in quantifying them (see Bourke & Franks 1995 for a review).

Previous research has shown low effective paternity, 1.11, in the European hornet *Vespa crabro* (Foster *et al*. 1999), leading to the prediction of queen–worker conflict over male production. As in all vespines, hornet workers are smaller than the queen and unable to mate but retain the ability to lay haploid male eggs in queenless groups (Matsuura & Yamane 1990). To test for worker reproduction in the queen's presence we used a novel combination of genetics, ovary dissection of workers and observation. In contrast to prediction, the data show an absence of any expressed conflict with the queen producing all the colony's males.

Methods

Thirty-two hornet nests were collected from the New Forest, Hampshire, UK in two collections in 1998, one in July and early August (ergonomic phase of the annual life-cycle) and the other in September (reproductive phase). Colonies are founded in May and end their annual life-cycle from September to November (Archer 1993). All nests were collected from pest control calls and would otherwise have been destroyed. Twenty were relocated to nest boxes for observation or maintenance at the Institute of Terrestrial Ecology, Furzebrook, Dorset. Twelve nests unsuitable for relocation, such as those lacking a queen or collected late in the season were immediately frozen at −70 °C.

Nest boxes

Wooden observation nest boxes were 9.5 cm (depth) × 30 cm (horizontal) × 40 cm (vertical) and were faced with a hinged glass door to allow observation and access. Eight were set up in a shed with plastic pipe (Ø 3 cm) to the outside allowing the hornets to forage in the wild. Ten wooden maintenance boxes, 20 cm × 20 cm × 40 cm with a 3-cm entrance hole in the side, were also made and nailed to trees in a wood. These allowed young colonies to become reproductive to provide samples of males.

Relocation

During relocation nests were chilled with ice and the workers separated from the combs. The combs, with the queen, were placed on horizontal wires inside the nest box. The chilled workers were then replaced and given 50 mL of honey to provide food during nest re-establishment. After about an hour the entrance to the nest box was opened and the hornets allowed to fly. Ten nests were relocated to observation boxes and 10 to maintenance boxes. Four observation and two maintenance nests remained queenright and developed to reproductive status.

Observation

The four observation nests were sequentially scan sampled for a total of 50 h (1956 scans), a maximum of 3 h per day, from 26 August to 28 September. Nest-envelope paper that prevented observation of the combs was gently removed at intervals by sliding a hacksaw blade behind the observation box door. In each scan, all combs were carefully examined noting queen position (comb number) and activity (oviposition, queen–worker or worker–worker aggression). An additional queenless nest was also observed to determine the duration of a worker oviposition. Five worker and five queen ovipositions were timed to give means of 126 and 130 s, respectively. The mean time taken to scan a nest was 92 s so that each nest was not watched for an average of 276 s during each round of scanning. On average, therefore, there was a 150-second period (276–126 s) each round when worker oviposition events would be missed. Thus, the effective period during which worker oviposition could be observed was approximately 118 h (218/368 × 50 h × 4 nests).

Genetic methods

Ten workers and the queen, if collected, from each of 19 nests were analysed at four DNA microsatellite loci using primers designed for *Vespula rufa* (Thorén 1998) and modified for use on *Vespa crabro* (Foster *et al*. 1999). Maternity was then assessed in up to 20 males at one or two loci for 13 nests. Loci were chosen in which the workers' paternal and maternal allele were different (informative genotypes). This allows identification of a worker-produced male by its inheritance of the mother worker's unique paternal allele (see also *Male nondetection error*). For five nests that had male pupae, equal numbers of male pupae from each comb were analysed instead of adult males in case this could give additional data on laying location. Males from two additional nests, collected in 1997, were also analysed. One of these nests was unusual in that some workers had activated ovaries (see Results). To raise the detection probability in this nest, 50 males were analysed. For the other nest 20 males were analysed to give a total sample of 282 males from 15 nests.

Worker–worker relatedness

Regression relatedness among offspring females (workers) (b), inbreeding (F), and allele frequencies were estimated from the worker-genotype frequency data using the program Relatedness 4.2 (Goodnight & Queller 1994). The program calculates standard error estimates for b and F by jackknifing across nests. Pedigree estimates of relatedness were made by inspecting worker genotypes across the four loci for each nest. This was used to produce a second estimate of relatedness (r), assuming outbreeding, and to estimate sperm bias when multiple paternity occurred.

Effective paternity

Effective paternity (M_e) was estimated from Pamilo (1993):

$$M_e = \frac{1}{2b - 0.5} \tag{1}$$

where b is the regression relatedness from Relatedness 4.2.

Male nondetection error

Workers' sons are only detected if: (i) the queen and her mate(s) have different alleles (an informative genotype); and (ii) they inherit the worker's paternal allele. With fair meiosis, the paternal allele is transmitted with probability 0.5. Hence, even if worker genotypes are informative, 50% of worker-produced males cannot be distinguished from queen-produced males at this locus. With unlinked loci, the total number of assignable males in a sample (N_a) can be estimated from:

$$N_a = \sum_j^n \left(1 - \prod_i^{l_j}(1 - 0.5p_{ij})\right) N_j \tag{2}$$

Where l_j is the number of loci and N_j the number of males analysed for the jth of n nests and p_{ij} is the proportion of informative worker genotypes at the ith loci of the jth nest. Weighting by p_{ij} assumes that workers of all genotypes are equally likely to reproduce.

Male nonsampling error

If workers produce a proportion x of the males, the probability of not sampling any worker-produced males is $(1 - x)^{Na}$.

Worker ovary activation

The ovaries of 500 workers from eight queenright nests were examined by dissection under a binocular microscope with a graticule eyepiece. The size of the largest egg was compared to the mean size of five worker-laid eggs (taken from a queenless nest) and placed into one of three categories: less than half size, greater than half size, greater than 90% full-size.

Results

Queen loss in the wild

Four of 23 prereproductive nests collected in July and early August were queenless and, hence, unable to develop to a large size. Pre-reproductive status was defined by the presence of worker-sized cells only (the start of large cell construction signifies the start of laying of gyne-destined and the great majority of male-destined eggs; Archer 1993). Two out of the nine reproductive nests collected in late September were queenless male-producing nests with reproductive workers.

Observations

Eighty-five queen ovipositions but no worker ovipositions were seen during scan sampling of the four queenright observation nests. The queen was completely ignored by the workers and no aggression between the two was ever seen. There were nine cases of workers mauling each other, an enigmatic behaviour seen in several vespine species (Greene 1991). The queen in all nests spent most time on the middle combs and least on the upper. The four nests observed all produced males and built at least one new comb after relocation. One nest produced approximately 150 workers, 100 males and 20 gynes. The other three nests produced about 50 workers and 5–10 males. This is within the natural range, but small.

Allelic diversity

Genetic variation at the four microsatellite loci studied was moderate, with 3–7 alleles per locus and a mean expected heterozygosity across all loci of 0.63 (Table 1). The allele frequencies did not significantly differ from the estimate obtained from 14 nests from the same population in 1997 (Foster *et al*. 1999) ($\chi^2 > 0.05$ for each locus).

Worker–worker relatedness

Worker nestmates were related by $b = 0.67 \pm 0.06$ SE across the 19 nests with an inbreeding coefficient not significantly different from zero $F = -0.063 \pm 0.065$. The pedigree estimate of worker nestmate relatedness gave a very similar result $r = 0.68 \pm 0.03$. Fourteen nests were monogynous and monoandrous, two nests had two matrilines and three had two patrilines. In the two patriline nests, the majority males fathered 95%, 60% and 50% of the workers analysed. In the two matriline nests, the majority matrilines represented 70% and 60%. An estimate of relatedness reflecting paternity effects was only obtained by weighting all matrilines equally, $b = 0.73 \pm 0.04$. This gives an effective paternity (M_e) of 1.04. Nondetection and nonsampling errors may cause relatedness to be slightly overestimated. However, the potential effects in this system are minor (Foster *et al*. 1999) and do not affect the key conclusion that M_e is much less than two and the prediction that worker reproduction is expected but worker policing is not (Ratnieks 1988).

Table 1 Genetic variation in the microsatellite marker loci studied, where n is the number of alleles detected in the 19 study colonies and H_E is the expected heterozygosity at each locus

Locus	n	Allele frequencies	H_E
5	7	0.133, 0.314, 0.008, 0.026, 0.147, 0.346, 0.026	0.74
13	3	0.600, 0.058, 0.342	0.52
15	5	0.108, 0.434, 0.250, 0.176, 0.032	0.71
18	4	0.060, 0.134, 0.614, 0.193	0.56
Mean	4.75		0.63

Male production

All haploid male genotypes from the 14 nests were consistent with being queens' sons. The number of assignable males N_a was estimated to be 176 (equation 2). This is equivalent to a probability of less than 5% of missing a worker contribution to male production greater than 2% (Fig. 1).

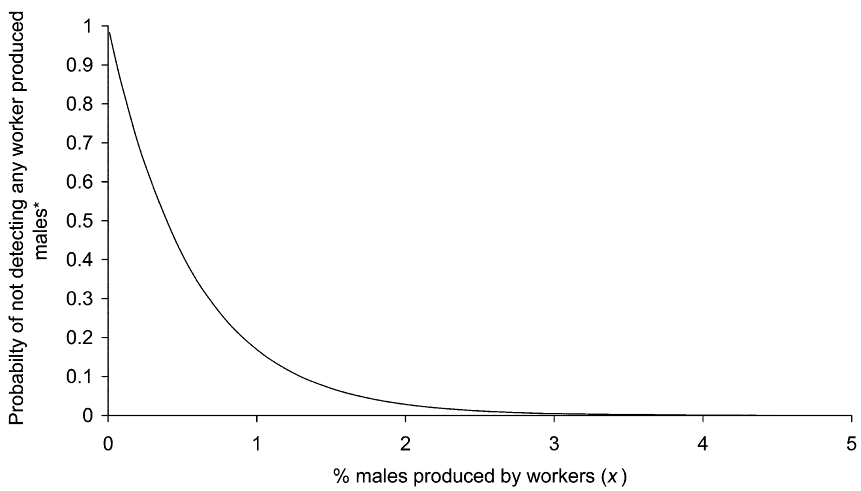

Fig. 1 Probability of not detecting any worker produced males as a function of the percentage of all males that are worker* produced, for 176 assignable males (N_a).

Diploid males

In one nest, which had a single patriline, the 10 males analysed were diploid. This nest consisted of the queen, 40 workers and 36 males and was notable in producing adult males at an early date, 8 August. These males were not counted in calculating N_a (see Methods).

Worker ovary activation

In 400 workers dissected from seven nests none had full-size eggs and only two had eggs greater than half size. In one nest 6/100 workers had a single full-size egg and another six had an egg greater than half size.

Discussion

The discovery of diploid males in *Vespa crabro* is the first record for the Vespinae (yellowjackets and hornets). Diploid males have been reported in many hymenopteran taxa but there are only two other records for social wasps, one Polistinae, and one Stenogastrinae (J. E. Strassman, personal communication in Crozier & Pamilo 1996). In the honeybee (*Apis mellifera*) and parasitoid wasp (*Bracon hebetor*) diploid males occur as a result of matched mating at a single multiallelic sex-determination locus (see Crozier & Pamilo 1996). A single sex-determination locus is consistent with our data in that there were near equal number of workers to males (40 to 36), as expected with single paternity assuming that diploid males have similar survival to females.

The worker–worker relatedness of 0.68 is very similar to the estimate of 0.70 obtained by an analysis of 14 nests from the same population in 1997 (Foster *et al*. 1999). In addition there is no significant difference in the proportion of multiple-patriline nests found (3/19 in 1998 vs. 5/14 in 1997, χ^2, $P = 0.307$). However, the discovery of two nests with two matrilines is new. Both nests had only one queen at collection, suggesting successful nest takeover by usurping queens. The data show that successful takeover is quite rare, occurring in only 2/33 nests (combining the 1997 and 1998 data). Nixon reported 'piratical' behaviour in queen hornets but found that usurped nests rapidly declined (Nixon 1983, 1986). The nests studied here had adult workers from the new queen showing that the colonies had survived at least one month since usurpation.

Queens of *V. crabro* produce all or the vast majority of the colonies' males. Genetic analysis of 272 haploid males from 14 nests ($N_a = 176$) revealed that all male genotypes were consistent with being queens' sons. Observational data further revealed an absence of any behavioural conflict between the queen and the workers, nor was there any attempted worker laying during an effective observational period of 118 h in which 82 ovipositions by queens were seen. This contrasts with the observation of worker laying in the queenless nest and data from a queenright *Dolichovespula arenaria* nest with 32 queen to 10 worker ovipositions (Greene *et al*. 1976). The two studies are comparable, with observations in both being made in the latter part of the reproductive period. Worker ovary dissection of *V. crabro* also suggests a general absence of worker reproduction, although one nest had some workers with activated ovaries indicating that worker laying in queenright colonies could occur. However, the 50 males analysed from this nest ($N_a = 37.5$) revealed no evidence of worker reproduction showing that, as in the other colonies, it is either absent or rare. In addition, ovary-activated workers only ever had one full-size egg in their ovaries suggesting that they would have had lower fecundity than the queen. Our data agree with other *Vespa* data. There are no confirmed reports of worker laying in queenright hornet colonies despite several observational studies (notably Nixon 1985; Matsuura & Yamane 1990). Ishay (1964) stated 'it was permissible to suppose' queenright worker reproduction occurred in the upper combs of *V. orientalis* since the queen spent most time on the lower combs, but no supporting data were given. In *V. analis*, only one out of 1062 workers from 16 queenright reproductive colonies had fully activated ovaries (Matsuura 1984). Martin (1990) dissected 600 workers from six *V. simillima* nests (500 from one nest) and found six workers with activated ovaries. However, as in this study, the activated ovaries only contained a single full-size egg contrasting with the ovaries of workers from queenless colonies, which contain several full-size eggs (SJ Martin, personal communication).

The absence of male production by workers in *V. crabro* does not fulfil our prediction arising from relatedness theory (Hamilton 1964). This contrasts with available data from *Dolichovespula*, and *Vespula*, where relatedness seems a reliable predictor of the absence or presence of male production by workers in queenright colonies (Ratnieks 1988; Foster *et al*. 1999). In *D. arenaria*, which has a similar low paternity, workers in queenright colonies have activated ovaries, oviposit and succeed in producing males in queenright colonies (F. L. W. Ratnieks and J. J. Boomsma, unpublished; Greene *et al*. 1976). In addition to kin structure, *V. crabro* and *D. arenaria* also share lifecycle, ecology, queen–worker size dimorphism and colony size making it hard to explain why worker reproductive behaviour should differ. If worker reproduction is costly to colony productivity, self-restraint (possibly enforced through worker policing) can be favoured (Ratnieks & Reeve 1992). However, with *D. arenaria* and *V. crabro* being so similar, there is no obvious reason for an increased cost to worker reproduction in *V. crabro*. This suggests either that an increased cost is caused by some subtle and as yet unknown factor or that such costs are not important.

An alternative hypothesis to explain the absence of worker reproduction in *V. crabro* is that the queen controls worker reproduction (Bourke & Franks 1995). Physical queen control (queen policing) is a likely explanation for the absence of worker reproduction in the small colony vespids *Polistes bellicosus* and *P. dorsalis* (Arevalo *et al*. 1998). However, no queen aggression or oophagy has been seen in *Vespa* (this study, Nixon 1985; Matsuura & Yamane 1990). Instead, hornet queens may exert indirect pheromonal control to cause the worker's acquiescent zombie-like behaviour. Queen pheromone (Ikan *et al*. 1969; *V. orientalis*) and royal courts (Nixon 1985; Matsuura 1991; *V. crabro*) both occur in *Vespa* and are not recorded in *Dolichovespula* (or *Vespula*, where queens are multiply mated and worker policing may act). Although queen pheromones may yet be discovered in these genera, studies looking for pheromones have not been successful (Greene 1991) and the absence of royal courts suggests that, if queen pheromones do occur, they have a less direct effect on workers.

The idea of queen pheromonal control has been criticised (Seeley 1985; Keller & Nonacs 1993). If the queen's signal is against worker interests then workers will be selected to ignore it, rendering it simply an honest signal of the queen's presence (Seeley 1985). However, the queen may then be selected to regain control and enter an arms race with the workers (West-Eberhard 1981). Keller & Nonacs (1993) argued that this would not persist, as it would quickly become too costly for the queen to invest in new and greater quantities of chemicals to prevent worker evasion. However, an arms race need not be costly to the participants. With no memory in the system, it can proceed by alternating between a limited set of states with little innovation and no escalation (p. 67 Ridely 1993; Lythgoe & Read 1999). In addition, the queen may have an inherent advantage in the race because workers in queenright colonies should only lay during the reproductive phase of the life cycle. Hence, in annual societies, for most of the season a queen signal would be cooperative and honest. Worker counter-evolution would be constrained because worker reproduction too early in the season will reduce the total amount of reproduction by the colony and be costly. The workers require a strategy that not only blocks worker response to queen pheromone but also only does so at a particular stage in the season. A persistent arms race is therefore a possibility. Providing evidence for or against such hypotheses is extremely difficult. However, one prediction of arms race theory is that the outcome should be fairly arbitrary across lineages (Bourke & Franks 1995, p. 239). This could explain why worker reproduction is absent in *V. crabro* but present in the otherwise similar *D. arenaria*.

In annual colonies with queen control, another strategy enabling workers to reproduce is matricide (Bourke 1994). There are several anecdotal reports of queen killing by workers in *V. crabro*. Nixon (1985) reported that at the peak of colony development workers may surround the queen and aggressively jostle her, but that the queen survived this attention. Matsuura (1984) described this as royal court behaviour, further stating that workers actually bite the queen and may kill her. Other possible accounts of matricide come from Janet (1895) who described a *V. crabro* worker biting the queen who later died and Ishay (1964) who stated that queens of *V. orientalis* are licked to death. However, only two out of nine colonies collected at the end of the season in this study were queenless nests and potential candidates for matricide. In addition, no aggression towards the queen was seen in the four observation colonies. Therefore, if matricide is a real phenomenon in *V. crabro*, it is probably restricted to a minority of colonies.

Kin selection predictions are complicated by unknown costs and constraints, which act in addition to the effects of relatedness. However, the comparison of similar species, such as *V. crabro and D. arenaria*, eliminates many potential variables and enables possible causal agents to be identified. This approach is important for the future of kin-selection research. It improves on the potentially anecdotal nature of single-species studies while being more specific than broad correlation (Ratnieks 1988; JE Strassmann, personal communication). Such comparisons require that the otherwise similar species differ in key reproductive traits. Several examples of this are found in the vespine wasps. Worker reproduction has been observed in *Vespula consobrina* (Akre *et al*. 1982) and *V. acadica* (Reed & Akre 1983) but not *V. atropilosa* (Akre *et al*. 1976), which are all members of the small colony *V. rufa* species group of *Vespula* (Carpenter 1987). Relatedness itself varies between and within species in the *D. norwegica* species group with single paternity found in most *D. norwegica* and *D. sylvestris* colonies but a mix of single and multiple paternity in *D. saxonica* (KR Foster and RLW Ratnieks, unpublished). Finally, royal court behaviour like that found in *Vespa* has been observed in the fourth vespine genus *Provespa* (Matsuura & Yamane 1990) allowing further investigation of the queen control hypothesis. This diversity of social traits in an otherwise homogenous group makes the vespine wasps an excellent group for further study of kin selection.

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This study forms part of Kevin Foster's ongoing PhD on 'Kin selection and reproductive conflict in vespine wasp societies'. The Laboratory of Apiculture and Social Insects at Sheffield is headed by Francis Ratnieks and has interests in social evolution, behavioural ecology, work organization in insect societies and apiculture. Alan Raybould's interests are the genetic structure and dynamics of natural populations, particularly with respect to plant–insect interactions.
