

Research article

## Low relatedness and frequent queen turnover in the stenogastrine wasp *Eustenogaster fraterna* favor the life insurance over the haplodiploid hypothesis for the origin of eusociality

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**Summary.** The origin of sociality is best studied in taxa with rudimentary social development, like the stenogastrine wasp, *Eustenogaster fraterna*. Our study of colony structure and relatedness in this species found very small colonies averaging only 2.7 adult females, including a single mated, reproductive female. Microsatellite genotyping showed that adult females were related to each other but not generally as full sisters ( $r = 0.427 \pm 0.131$ , 95% confidence interval). Microsatellite genotyping showed that sociality is not favored by the haplodiploid hypothesis, because relatedness of unmated females to female brood is low ( $r = 0.210 \pm 0.171$ , 95% confidence interval), far lower than that among sisters in either haplodiploid or diploid species. Relatedness of unmated females to female brood is significantly lower than that of mated females to female brood ( $r = 0.374 \pm 0.266$ ). Mated females are also significantly more related to the male brood ( $r = 0.871 \pm 0.168$ ) than are unmated females ( $r = 0.588 \pm 0.339$ ), suggesting that unmated helpers do not generally produce sons. These results argue against an important role for exceptionally high relatedness in the origin of eusociality. One quarter of the brood could not have been the progeny of any collected female, suggesting high rates of queen turnover. In all, 7/17 nests had some brood that could not be assigned to existing adult females. These high adult mortality rates result in direct advantages to helpers in the form of colony inheritance, and indirect advantages via life insurance benefits.

**Key words:** Life insurance; microsatellite; relatedness; usurpation, Vespidae.

### Introduction

Cooperation is particularly amenable for study in primitively social species where all females retain the options of helping or reproducing. Hamilton's Rule gives the thresholds under which helping can be favored:  $B/C > 1/r$ , where B is the relative's gain of offspring, C is the helper's loss of offspring she could have reared independently, and r is the helper's relatedness to the young produced because of her aid (Hamilton, 1964a). Helping may be favored by high relatedness among helpers and brood, a circumstance that would require only modest benefits of helping relative to reproducing (Hamilton, 1964a, b, 1972; Bourke and Franks, 1995). Helping may also be favored because of high benefits of helping relative to the amount of lost reproduction that would have been achieved alone (e.g. Alexander, 1974; West Eberhard, 1975; Evans, 1977; Strassmann and Queller, 1989; Alexander et al., 1991; Queller and Strassmann, 1998). Once relatedness within colonies is known, the magnitude of benefits relative to costs necessary to favor helping becomes more clear.

Alternatively, altruists might achieve direct benefits by inheriting colonies and their workforces, or they could gain indirect fitness benefits by helping nondescendent kin (Hamilton, 1964a, b). In the Hymenoptera, indirect benefits are likely to come from life insurance advantages (Strassmann and Queller, 1989; Queller, 1989; 1994a; 1996; Queller and Strassmann, 1998; Field et al., 1999). This is because high mortalities of foraging females combined with long offspring dependence times favor grouping as a means of insuring offspring reach adulthood. However, if the inheriting female is unrelated to the brood, it will only benefit her if those brood eventually help rear her brood.

The focus of this study is *Eustenogaster fraterna* van der Vecht, a member of the tribe Stenogastrini. The stenogastrine wasps of southeastern Asia are just past the threshold of eusociality. They have very small colonies with apparently

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totipotent females. For example, adult colony members typically number under 5 in *Parischnogaster mellyi* Saussure and *Parischnogaster striatula* du Buysson (Turillazzi, 1996), as many as 7-8 in *Liostenogaster flavolineata* (Samuel, 1987) and 13 in *P. alternata* (Turillazzi, 1989, 1996). A single wasp typically dominates reproduction in a colony and prevents other nestmates from laying eggs, but no morphological caste differences separate females (Turillazzi, 1989; 1996; Sumner et al., 2002). Usually, the egg-layer spends most of her time on the nest while non-laying females help with brood care and forage. These subordinate females are typically reproductively competent and can mate and become queens should a vacancy at the top arise (Turillazzi, 1991; Field and Foster, 1999). The very long brood development times and high adult mortality rates mean that vacancies in queenship do occur (Samuels, 1987; Field et al., 2000). Helping in their natal colony is not the only option for subordinate females. They can also join another colony as a subordinate, or even as a dominant if they succeed in defeating the other females (Yamane et al., 1983; Turillazzi, 1989). Joining other colonies is facilitated because stenogastrine wasps also often nest in aggregations dense enough to present females with a variety of reproductive options (Samuels, 1987; Turillazzi, 1996; Field and Foster, 1999).

Relatedness among female colony members has rarely been measured but was found to be low in the tribe compared to the potential maximum of 0.75 relatedness between sisters. In *Liostenogaster flavolineata* low relatedness ( $r = 0.22 \pm 0.10$  and  $r = 0.52 \pm 0.05$  in another study) was reported and attributed to high usurpation rates by unrelated females (Strassmann et al., 1994; Field et al., 2000). In this species, helpers are critical for ensuring that immature brood become

adults because of the combination of high adult mortality and exceptionally long egg-to-adult developmental times averaging more than 100 days (Field et al., 2000; Samuel, 1987).

We studied colony structure and relatedness in *Eustenogaster fraterna*. This species builds small mud nests with a single entrance hole underneath, and typically has colonies with fewer than five females (Turillazzi and Gerace, 1992; Francescato et al., 2002). It is particularly interesting because the division of labor between the egg layer and the subordinates is slight, with subordinates apparently working no more than dominants (Francescato et al., 2002).

**Material and methods**

*Collection techniques*

In February 1999 we collected 18 colonies of *E. fraterna* in the tropical rain forest at Bukit Fraser, Pahang State, Malaysia, at an altitude of 1000–1500 m. We collected in the evening (18:30 to 19:30) and at night (20:30 to 21:00). Evening collections facilitated getting all the wasps on difficult-to-collect colonies but at a possible cost of missing late returning foragers. However in both evening and night collections we got all wasps on the nest and no flying foragers were observed. Colonies were refrigerated at 4°C and the nest morning adults, eggs, larvae, and pupae were transferred to plastic tubes containing 100% ethanol that was changed twice.

*Ovary, spermathecae and size status*

In order to identify the reproductives in each colony, we dissected the ovaries of all females (Table 1). We counted the number of mature and nearly mature eggs and we measured the length of the longest oocyte. A mature egg is at least as long as the smallest egg laid (1.6 mm) and has

**Table 1.** Colony characteristics. Rows are sorted by number of adult females.

Nest ID	No. adult females	No. adult males	No. females lacking mature eggs	No. females w/ mature eggs	No. females w/ nearly mature but no mature eggs	No. mated females	No. Cells	No. Eggs	No. Larvae	No. Pupae	No. Empty Cells
17	1	0	0	0		*	12	4	1	5	2
1	1	0				*	13	3	0	1	9
2	1	0				*	13	9	0	0	4
16	1	0	0	0	0	0	16	9	5	0	2
18	1	0	0	0	1	0	18	9	3	0	6
7	1	0	0	1'	0	1	23	6	6	0	11
14	2	0	1	1'	1	1**	12	3	2	7	0
6	2	0	2	0	1'	1	14	6	2	0	6
4	2	0	0	1'		1*	20	5	6	2	7
3	3	0	2	1'	0	1	9	6	0	0	3
10	3	0	2	1'	1'	2	12	5	1	0	6
8	3	0	1	1'+1	1	1	15	1	0	0	14
12	3	0	1	1'+1	1	1	23	12	3	0	8
11	4	1	4	0	2	0	12	6	4	0	2
5	4	0	2	1'+1	0	1	17	9	6	0	2
15	5	0	3	1'+1	2	1***	16	7	2	0	5
9	6	1	2	1'+3	0	1	17	6	1	1	9
13	6	0	5	1	3	**	19	15	2	0	2

Nests 11–15 collected at night. ' mated; \*one female lost abdomen; \*\*one female lost spermatheca; \*\*\* two females lost spermatheca.

a clear chorionic membrane. A nearly mature egg typically is 80% or more of the length of a mature egg, is opaque and lacks the final chorionic membrane. We also determined whether the wasps were mated by checking for sperm in the spermatheca. We were unable to assess insemination status from 3 females and for 4 additional females we could not assess insemination or ovarian status because we lost the abdomen. These problems affected 7 colonies, three with a single queen (Table 1). We measured headwidths to determine whether reproducers were larger than non-reproducers. We used a graticule-equipped Wild microscope at 25× for the dissections and morphological measurements.

### Molecular techniques

We extracted DNA from the thorax tissue of adults and pupae, and a similarly-sized piece of larvae, using a BIO-RAD CHELEX-100 extraction (Hillis et al., 1996). For genotyping, we used the polymerase chain reaction (PCR) to amplify five microsatellite loci, identified from *Eustenogaster fraterna* (Table 2; Zhu et al., 2000). The number of alleles ranged between 7 and 14, with observed heterozygosities of 0.47–0.82 (see Strassmann et al., 1996, for detail on the procedures for the amplification of microsatellites and resolution of PCR products).

The genetic data were generally not adequate for constructing detailed pedigree connections because of the small numbers of adults and brood, the absence of many mothers, lack of information as to whether females mate multiply, and the modest number of microsatellite loci. Therefore we focused on two modes of analysis that were more robust. We estimated average relatednesses among categories of individuals, and we determined how often we can exclude all adult females as mothers, as a possible indicator that the mother(s) had died.

### Relatedness estimations

We estimated genetic relatedness using Relatedness 5.0.5 (Queller and Goodnight, 1989), weighting colonies equally. In the analysis, we

**Table 2.** DNA microsatellite primers and their frequencies in this population.

Primer	Length	Frequency	Primer	Length	Frequency
Ef98AAG	241	0.094	Ef213AAT	230	0.125
	244	0.309		233	0.149
	247	0.173		236	0.054
	250	0.2		239	0.576
	253	0.159		242	0.008
	256	0.053		245	0.082
	259	0.012		248	0.006
Ef103AAG	249	0.061	Ef217GA	199	0.069
	252	0.173		205	0.016
	255	0.275		213	0.117
	258	0.097		217	0.171
	261	0.141		221	0.065
	264	0.209		223	0.157
	267	0.044		225	0.067
				227	0.004
Ef184AAC	110	0.011	233	0.011	
	113	0.239	235	0.076	
	116	0.434	237	0.028	
	119	0.097	239	0.211	
	122	0.133	241	0.008	
	125	0.014			
	128	0.016			
	134	0.013			
	137	0.008			
	140	0.037			

excluded individuals with genotypes for fewer than three microsatellite loci. This affected brood only. We jackknifed over colonies in order to estimate 95% confidence intervals and these are the values reported with all relatedness estimates. We compared relatednesses with unpaired tests, because pairing the nests would reduce the sample size too much (Queller, 1994b). We classified brood as male if they had only one allele at all loci, indicating they were haploid. The chance that a female would be homozygous at all loci was only  $5.33 \times 10^{-4}$  so we do not think we assigned any brood incorrectly to the male class.

We determined which brood could be excluded as progeny of all possible mothers in their colony. We excluded female brood if they did not share at least one allele at every locus with a potential mother, where potential mothers are mated females only. If mothers were missing abdomens or spermathecae, we assumed they could have been mated and treated them as potential mothers, so our analysis is conservative with respect to the number of offspring excluded. We assigned sons to possible mothers by requiring that they share an allele at all loci with their mother, who did not have to be mated. We counted missing loci as possibly matching since we could not determine otherwise. This also has the conservative effect of increasing the number of brood assigned to present females.

## Results

### Colony characteristics

Nests of *E. fraterna* were small, with cell number ranging from 9 to 23 (average =  $15.6 \pm \text{SD}3.9$ ,  $N = 18$ , Table 1). Most occupied cells contained eggs ( $6.7 \pm \text{SD}3.4$ ,  $N = 18$ ). Fourteen of these nests also contained larvae ( $2.4 \pm \text{SD}2.2$ ,  $N = 14$ ) and 5 of these nests also contained pupae ( $0.9 \pm \text{SD}2.0$ ,  $N = 5$ ). Seventeen nests had empty cells ( $5.8 \pm \text{SD}3.6$ ,  $N = 17$ ). Subsequent genotyping indicated that from twelve nests some of the brood were males ( $2.1 \pm \text{SD}1.2$ ,  $N = 12$ ; Table 3).

Nests had few adults on them (Table 1). Overall they averaged  $2.7 \pm \text{SD}1.7$  adult females. Two of the larger nests (4 and 6 females) each had a single adult male. The number of mated females did not differ between evening ( $0.9 \pm \text{SD}0.6$ ,  $N = 10$ ) and night collections ( $0.6 \pm 0.5$ ,  $N = 5$ ; Mann-Whitney U test,  $p > 0.4$ ). The number of unmated females also did not differ between evening ( $1.8 \pm \text{SD}1.5$ ,  $N = 9$ ) and night collections ( $3.3 \pm \text{SD}1.5$ ,  $N = 4$ ; Mann-Whitney U,  $p > 0.1$ ) though there was a trend towards lower numbers in the evening indicating we may have missed some foragers.

### Female characteristics

In our sample 37% of females had mature eggs, and 24% of females were mated (Table 1). Among mated adults, 10 of 12 (83.3%) had mature eggs, while only 8 out of 29 (27.6%) non-mated females had mature eggs in their ovaries (G-squared Test = 11.25,  $p < 0.001$ ). These unmated egg layers do not form a distinct class of females because other females had ovaries nearly developed to that level (Table 1). The difference in length of the longest oocyte present in ovaries of egg-layers and non-egg-layers was significant ( $t$ -test,  $t = 7.004$ ,  $P < 0.0001$ , Table 1). Most colonies had more than one female with mature or nearly mature eggs in her ovaries,

**Table 3.** Numbers of full sister groups among adult females, proportion of adult females that could be sisters or daughters of the mated egg layer, and the proportion of brood that could be assigned to these adults. Female brood could be assigned only to mated females while male brood could be assigned to any adult female. Data are based on analysis of microsatellites (see methods). Rows are sorted by number of adult females.

Nest	No. adult females genotyped	No. adult sister groups	Proportion adults not sisters or daughters	No. female brood genotyped	Prop. brood not daughters	No. male brood genotyped	Prop. brood not sons
17	1	1	–	1	0	3	0
1	1	1	–	1	1	0	–
16	1	1	–	6	0.17	1	1
18	1	1	–	1	0	1	0
7	1	1	–	3	0	2	0
14	2	1	0	6	0	4	0
6	2	1	0	1	1	0	–
4	2	1	0	4	0.25	3	1
3	3	2	0.5	0	–	0	–
10	3	2	0.5	1	0	0	–
8	3	2	0	0	–	0	–
12	3	1	0	2	0	1	0
11	4	3	*	3	1	1	1
5	4	2	0	2	0	4	0
15	5	2	0	1	0	2	0
9	6	4	0.8	1	1	1	1
13	6	2	*	1	1	2	0

\* No dominant mated egg layer identified made these impossible to categorize.

though only one of them was mated in any given colony as far as we were able to determine. In one colony two females of three were mated, but only one of them had mature eggs in her ovaries and this is the only case where there was more than one mated female in a colony.

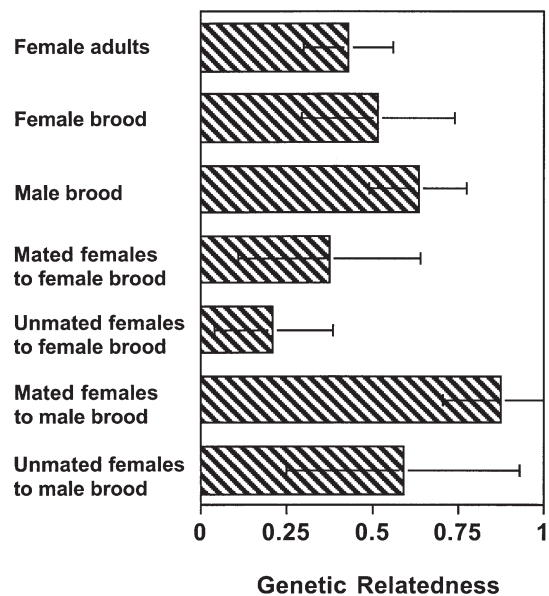
Three colonies lacked a mated egg layer. Two of these had only a single female, in one case with undeveloped ovaries, and in the other case with 3 nearly mature eggs. The third colony lacking a mated egg layer had 4 females, one with a mature egg, and all with some ovarian development. On four other colonies we could not be sure about the presence of a mated egg layer since we lost the spermatheca or abdomen of at least one female.

Size did not differ between reproductive females and non-reproductive females. The average headwidths for mated and unmated females was  $1.403 \pm \text{S.D.}0.044 \text{ mm}$  ( $N = 12$ ) and  $1.397 \pm \text{S.D.} 0.046 \text{ mm}$  ( $N = 29$ ) respectively ( $t$ -test,  $p > 0.7$ ).

*Relatedness and parentage*

Average relatedness among adult females was  $0.427 \pm 0.131$  (12 colonies 43 females, Fig. 1). Mated and unmated females were related to each other by  $0.531 \pm 0.306$  (8 nests, 8 and 18 females, in the two classes respectively). Within colonies, adult females were related to female brood by  $0.272 \pm 0.11$  (15 nests, 42 adults and 33 female brood), and to male brood by  $0.673 \pm 0.211$  (12 colonies, 36 adults, 25 male brood).

Mated females were related to female brood by  $0.374 \pm 0.266$  (9 colonies, 10 and 20 females respectively), and to male brood by  $0.871 \pm 0.168$  (7 colonies, 7 and 17 individuals respectively, Fig. 1). Unmated females were related to female brood by  $0.210 \pm 0.171$  (10 colonies, 25 and 18 indi-



**Figure 1.** Genetic relatedness and 95% confidence intervals among listed categories. The top three bars indicate relatedness among the category listed. The last four bars indicate relatedness of the first category to the second category.

viduals respectively) and to male brood by  $0.588 \pm 0.339$  (8 colonies, 23 and 13 individuals respectively). Mated females were significantly more closely related to female brood than were non-mated females (unpaired  $t$ -test,  $p = 0.003$ ; 10 and 10 colonies). Mated females were also significantly more closely related to male brood than were non-mated females (unpaired  $t$ -test,  $p < 0.001$ ; 8 and 7 colonies).

Six of 31 female brood (19%) and 6 of 25 (24%) male brood could not have been the progeny of any of the adults we collected (Table 3).

## Discussion

Colonies were small and were tended by few females. Nevertheless, there was a reproductive hierarchy among the females tending these tiny nests. Each nest had only a single mated female who usually produced most of the brood, though some colonies had no brood produced by the dominant female or by other females, indicating the dominant female recently inherited the nest. The reproductive hierarchy does not extend to a behavioral hierarchy. Rank did not explain differences in time away from the nest among females (Francescato et al., 2002).

Facultative helping behavior grades into true eusociality when helpers have low probabilities of ever becoming reproducers (Sherman et al., 1995). Stenogastrine wasps stand on the threshold of eusociality, at a point where the selective factors favoring helping over reproducing can be analyzed. The haplodiploid hypothesis (Hamilton, 1964 a,b, 1972) offered the possibility of explaining eusociality purely on relatedness differences. However, this hypothesis has not been supported well here or by other empirical studies (Queller and Strassmann, 1998) even though its initial condition of single mating by queens holds widely across the social Hymenoptera (Strassmann, 2001). Multiple queens and frequent queen turnover are hallmarks of primitively social Hymenoptera and reduce relatednesses below that of full-sister frequently enough that the relatedness advantage of haplodiploidy often cannot apply (Queller and Strassmann, 1998).

*E. fraterna* is no exception. Genetic relatedness of unmated females to female brood was well below what would be found on colonies with single, once-mated queens, but significantly above zero. This could either be because of multiple mating by the queen, or because of queen turnover. Multiple mating is unlikely given its rarity in social Hymenoptera generally, and particularly in primitively social ants bees and wasps (Strassmann, 2001). Queen turnover is a more likely explanation for low relatedness. Unmated females could obtain direct fitness advantages by producing males, but our data suggest that this is not a major factor in *E. fraterna*. Relatedness of the mated females to male brood was very high, significantly above relatedness of unmated females to males, indicating the main egg layers also produce the males.

Relatedness among cooperating females of *E. fraterna* is between the two values previously reported for another stenogastrine wasp, *L. flavolineata* (Strassmann et al., 1994; Field et al., 2000). It falls above that reported for 4 *Polistes* species, and below the values reported for 8 other *Polistes* species making in unremarkable for primitively social wasps (Strassmann et al., 1989). Relatedness levels among cooperating stenogastrine females from this study and the two studies on *Liostenogaster flavolineata* indicate that low relatedness ( $r = 0.22 \pm 0.10$  and  $r = 0.52 \pm 0.05$  in another study)

was reported and attributed to high usurpation rates by unrelated females (Strassmann et al., 1994; Field et al., 2000).

There must be benefits to non-reproducers that outweigh the lower relatedness they have to brood as compared to what they would have to their own brood. These benefits are likely to take two forms. The first occurs if a solitary adult is likely to die before she can raise some of her brood to independence. Her effort is wasted. By contrast, a helper who dies early does not waste her contributions because she has completed the investments of other females or has her own investments completed by others. This is called the life insurance hypothesis (Strassmann and Queller, 1989; Queller, 1989; 1994; 1996; Gadagkar, 1990). The likely frequency of turnover combined with significant levels of relatedness among adults and brood and long brood development times, suggest that life insurance advantages are significant in *E. fraterna*. Brood development times in this species are likely to be similar to the 64 days reported for a close congener, *E. calyptodoma* (Hansell, 1987).

Second, helpers may also remain in colonies because they may inherit the nest and its workforce, a direct fitness advantage. High rates of egg layer replacement are a possible explanation for the unassigned brood. About a fifth of male and female brood could not be attributed to any adult female currently associated with the nest, and must have been offspring of uncollected, presumably dead, females, related or unrelated to the current females. This, together with the extremely small size of *E. fraterna* colonies, suggests inheritance probabilities for individual subordinate females are appreciable.

*Eustenogaster calyptodoma* is a closely related species studied by Hansell (1987) who observed frequent attempts at usurpation, which were sometimes successful. Usurpations were not followed by brood cannibalism, but instead the brood were adopted and cared for. In *E. calyptodoma* subordinate females associated with nests did not have ovarian development, though the unaffiliated categories "squatters and vagrants" had intermediate ovarian development (Hansell, 1987). Brood development times averaged 64 days for this species, shorter than the 105 days found for *Liostenogaster flavolineata*.

In another stenogastrine species, *Liostenogaster flavolineata*, with somewhat larger colonies in larger aggregations, Field et al. (1999) studied the reproductive options of females. They found that females went through a time window early in their adult life when they were likely to leave their natal nest and attempt to join another nest. Field et al. (1999) experimentally demonstrated that even females functioning as helpers can become queens, so poor condition is not likely to influence whether they help at home or float to a new nest they may be able to usurp (Field and Foster, 1999). The chance of becoming a reproductive is higher with smaller groups (Shreeves and Field, 2002). Though groups are small, reproduction is not contemporaneously shared: reproductive skew is high (Sumner et al., 2002). Earlier work had clearly demonstrated for this species that maturing brood were viewed as potential helpers, even by non-relatives, since nests with older brood were more likely to be adopted when

their adults were removed than were nests with younger brood (Field et al., 1998). Though *L. flavolineata* differs from *E. fraterna* in its larger group sizes, clumped nests, and longer brood development times, it is likely that similar selective factors operate in both species, favoring both moving among nests, and adopting unrelated brood as future caretakers of one's own brood. High adult mortality selects for sociality through inheritance, and through life insurance. These advantages appear to be important in *E. fraterna*, and may generally be what has favored the emergence of true sociality in species with extended parental care.

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