

# Relationship of queen number and queen relatedness in multiple-queen colonies of the fire ant *Solenopsis invicta*

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**Abstract.** 1. The relationship between the number of queens per nest and their relatedness was examined in the polygyne (multiple queens per nest) social form of the fire ant *Solenopsis invicta*.

2. No significant relationship between these two variables was found. Moreover, the overall average relatedness among nestmate queens was not significantly different from zero.

3. These findings indicate that polygyne nests of *S. invicta* do not represent closed societies. Furthermore, they are consistent with continual acceptance of non-nestmate queens throughout a colony's lifetime. This strategy differs from the expectation of kin selection theory that only related queens should be accepted as new reproductives within polygyne colonies.

4. The postulated acceptance of non-nestmate queens is associated with a decrease in the number of parasites, predators and diseases and a concomitant increase in the density of nests in introduced populations, suggesting that the permissive environment experienced by introduced fire ants may have decreased the relative importance of kinship as a stabilizing factor in the evolution of polygyny.

**Key words.** Fire ants, monogyny, polygyny, relatedness, social insects, *Solenopsis invicta*.

## Introduction

Genetic relatedness is believed to be an important factor in the development of sociality. In the haplodiploid Hymenoptera, female relatives of many sorts have elevated relatedness compared with equivalent classes of relatives in diploid taxa. This fact has been used to bolster kin selection as an explanation for both the origin and maintenance of worker sterility in the eusocial Hymenoptera (Hamilton, 1964a,b, 1972; Trivers & Hare, 1976; Hölldobler & Wilson, 1990; Bourke & Franks, 1995; Crozier & Pamilo, 1996). Many studies of kin selection in ants (family Formicidae) have measured relatedness among individuals within colonies (reviewed in Hölldobler & Wilson, 1990; Herbers, 1993; Rosengren *et al.*, 1993; Bourke & Franks, 1995; Crozier & Pamilo, 1996). Findings of high relatedness within a colony are consistent with the expectation that the often extraordinary levels of altruism found in ants (e.g.

obligate worker sterility) evolve through kin selection, whereas the discovery of very low relatedness appears to challenge the importance of kinship as a primary factor in ant social evolution. Therefore, factors that decrease relatedness between interactants, such as multiple nestmate queens, multiple mating by queens, or worker reproduction, potentially pose problems for kin selection theory (Hamilton, 1964b; Wade, 1978, 1982; Page & Metcalf, 1982).

In ants, the presence of stable multiple-queen colonies (polygyny) is widespread (Hölldobler & Wilson, 1977; Ross & Carpenter, 1991; Keller, 1993). Workers in polygyne colonies are likely to raise offspring of queens who are not their mothers, and thus they may not obtain indirect fitness benefits as large as those obtained by workers in colonies headed by a single queen (monogyny colonies). However, if queens within polygyne colonies are themselves highly related, then the workers that they produce may still raise close relatives and the predictions of kin selection theory may not be violated. Indeed, many studies measuring the relatedness of nestmate queens (hereafter referred to as queen relatedness) have found such queens to be close kin. However, colonies in other ants contain reproductive queens that, on average, have very low

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or even zero relatedness to one another (Hölldobler & Wilson, 1990; Herbers, 1993; Rosengren *et al.*, 1993; Bourke & Franks, 1995; Ross & Keller, 1995; Crozier & Pamilo, 1996). Such polygyne colonies may be evolutionarily unstable, because the altruistic behaviour of workers is vulnerable to the invasion of selfish alleles for reproduction (Crozier, 1979; Pamilo, 1991) or nepotistic alleles for discrimination that may lead to the breakdown of colony cohesion (Bourke & Franks, 1995).

Keller (1995) has recently synthesized and tested a hypothesis that may account for the low queen relatedness displayed by some polygyne ants. The first part of the hypothesis explains the evolutionary stability of such societies. Keller proposes that, because workers in such species are often obligately sterile, they may be 'trapped in their worker phenotype' and thus be making the 'best of a bad job' (Keller, 1995). That is, although workers are unable to augment their fitness directly by laying eggs, they may still obtain low indirect fitness benefits by raising distantly related nestmate brood. The second part of the hypothesis, which is the focus of this study, provides a proximate mechanism to explain the formation of colonies in these ants. A polygyne colony may begin with just a few closely related nestmate queens, and, as the colony ages, more reproductives are adopted. The newly accepted queens are recruited from within the colony, but they mate with non-nestmate males. Thus, over several generations, queen relatedness is eroded, even though queens are always adopted into their natal nests (Nonacs, 1988; Pamilo, 1991). As support for this proposed mechanism, Keller showed that a negative correlation exists between colony queen number and queen relatedness using both species- and population-level data. Such a correlation is consistent with a pattern of colony ontogeny in which the addition of queens over several generations leads to an inevitable decline in queen relatedness.

The species- and population-level data cited as evidence for the proximate part of the hypothesis do not provide as strong a test as would within-population data, because examination of relatedness and queen number across species does not directly address the issue of how polygyne colonies in single populations develop. Two recent studies investigating the relationship between queen number and queen relatedness within single ant populations found no significant relationship between these two variables (Evans, 1996; Herbers & Stuart, 1996), although both may have lacked the power to detect a significant trend. Queen number and its variance from nest to nest are low in both species studied, so it may be difficult to discern a significant association between queen number and queen relatedness. Furthermore, the high average queen relatedness found in these species implies that the postulated multigenerational mechanism of queen acceptance probably does not occur.

The red imported fire ant, *Solenopsis invicta*, may be a more suitable species for testing Keller's queen recruitment hypothesis. Most *S. invicta* colonies in North America contain a single reproductive queen. However, an increasing frequency of polygyne colonies has been found throughout the introduced range (Fletcher *et al.*, 1980; Ross & Keller, 1995) and, surprisingly, average queen relatedness within these colonies is near zero (Ross & Fletcher, 1985; Ross, 1993). Furthermore,

polygyne colonies in the introduced range of this species show extraordinary variation in queen number (Vargo & Fletcher, 1987; Ross, 1993). Circumstantial evidence for the hypothesized mechanism of queen acceptance exists because the number of queens per nest is lower and queen relatedness is higher in native, polygyne populations from Argentina, than in introduced polygyne populations (Ross *et al.*, 1996). This population-level correlation is expected if native polygyne colonies do not undergo the many rounds of queen acceptance that lead to substantial erosion of queen relatedness. However, considering the demographic and genetic changes that *S. invicta* has undergone upon introduction (Ross & Keller, 1995), it is doubtful whether this across-continent observation is useful for evaluating the hypothesis. Thus, it is more appropriate to examine the relationship between colony queen number and queen relatedness within a single population of introduced *S. invicta*. A finding of a negative relationship between colony queen number and queen relatedness would be consistent with the theory's posited mode of colony development, and would help to explain the persistence and spread of polygyne colonies in North America in the face of low nestmate relatedness.

## Materials and Methods

Seventy *S. invicta* nests displaying a wide range of sizes were collected from a polygyne population in Walton County, Georgia, U.S.A. To avoid sampling a colony fragment [a single nest of a colony that inhabits several nests (Bhatkar & Vinson, 1987)], which could yield incorrect estimates of queen number and relatedness, only nests that were separated from other nests by more than 2 m were collected. Thirty-one of the nests were collected from an area of  $\approx 4 \text{ km}^2$  in the spring of 1990 and thirty-nine from a different area of  $\approx 1 \text{ km}^2$  in the spring of 1994. All nests were collected before the first major mating flight of the season. Nest inhabitants were separated from the soil by flooding and were introduced into large trays (Jouvenaz *et al.*, 1977). Reproductive dealate (wingless) queens were collected by searching systematically through all ants in the trays. Nests were considered polygyne if they contained more than one dealate queen [wing-shedding is linked to the onset of oogenesis in this ant (Vargo & Laurel, 1994)]. In order to determine whether dealate queens had mated, their spermathecae were examined following dissection. Sperm in mated queens was evident as an opaque, whitish mass.

Standard electrophoretic methods (Shoemaker *et al.*, 1992) were used to determine the genotype of each collected queen at six polymorphic enzyme-encoding loci (*Aat-2*, *Acoh-1*, *Acoh-5*, *Est-4*, *G3pdh-1* and *Pgm-1*). Relatedness values for nestmate queens were estimated using the program RELATEDNESS 4.2 (Queller & Goodnight, 1989). Calculations were conducted using all queens, only mated queens, and only unmated queens. All individuals in both data sets were used to calculate background population allele frequencies ( $\bar{P}$ ) that are central to estimating relatedness with this algorithm, and groups (nests) were always weighted equally.

Because this study explores the association between queen number and relatedness values, it was necessary to avoid any

possible bias that would alter the relationship between these two variables. One possible source of bias may exist in the method by which relatedness values are calculated if, for instance, estimates of relatedness are slightly but consistently affected by queen number itself. To avoid any potential bias, a procedure was designed that controls for differences in group size when calculating relatedness. A resampling routine was conducted in which the genotypes of two individuals (the minimum number present in any nest) whose complete multilocus genotypes were known were chosen at random from each nest. Then, a within-nest relatedness value was calculated for this reduced sample using all queens to estimate  $\bar{P}$ . This procedure was repeated 100 times, and the arithmetic average of the relatedness values from the 100 trials was used as a corrected, unbiased estimate for queen relatedness in each nest. A corrected population-wide estimate of queen relatedness was obtained by taking the mean of the 100 population-wide values of average queen relatedness obtained from each bout of resampling. Standard errors of the means for the corrected relatedness estimates were taken to be the standard deviations for the 100 values of average queen relatedness obtained by resampling.

The samples collected from the different years were heterogeneous with respect to proportion of queens mated and allele frequencies at two of the six loci; therefore, the data were analysed separately by year as well as together. The separate analyses yielded similar results for all calculations, so only the analyses of the combined data are presented.

## Results

Twenty-eight of the thirty-one, and thirty-five of the thirty-nine nests in the 1990 and 1994 samples, respectively, were confirmed to be polygyne. The remaining nests contained either one or no queens, and thus were not included in further analyses. Figure 1 illustrates the frequency distribution of dealate queens per nest in the total sample of confirmed polygyne nests. Unmated queen number was found to be correlated with mated queen number using Spearman's rank order correlation coefficient ( $r_s = 0.620$ ,  $P < 0.001$ ,  $n = 63$ ), indicating that unmated queens constituted a relatively constant fraction of the queens in each nest.

Table 1 shows comparisons between corrected and uncorrected individual-nest relatedness values. The corrected relatedness estimates were found to be highly correlated with the uncorrected relatedness estimates (top row). Furthermore, the corrected population-wide mean estimate of queen relatedness was judged to be not significantly different from the uncorrected value (it falls within the 95% confidence interval of the uncorrected value). However, the uncorrected relatedness estimates obtained when considering total queens or only mated queens are biased upwards (Table 1, middle row). The relationship between the magnitude of the difference between the corrected and uncorrected relatedness values and the total number of queens per nest was examined to determine whether the uncorrected estimates systematically scale with group size in a fashion that could bias the results. A marginally

significant relationship was revealed using Spearman's rank order correlation test ( $r_s = 0.226$ ,  $P = 0.085$ ,  $n = 59$ ). Thus, it appears that use of uncorrected relatedness estimates might bias analysis of the relationship between queen number and relatedness. This bias is likely to be slight, however, because the slopes of the regression lines calculated for all nests using the two types of relatedness estimates did not differ significantly (Table 1, bottom row). Both sets of values lead to qualitatively equivalent conclusions with respect to the relationship of queen number and relatedness but, for the sake of accuracy in the point estimates, only results using the corrected relatedness values are considered further.

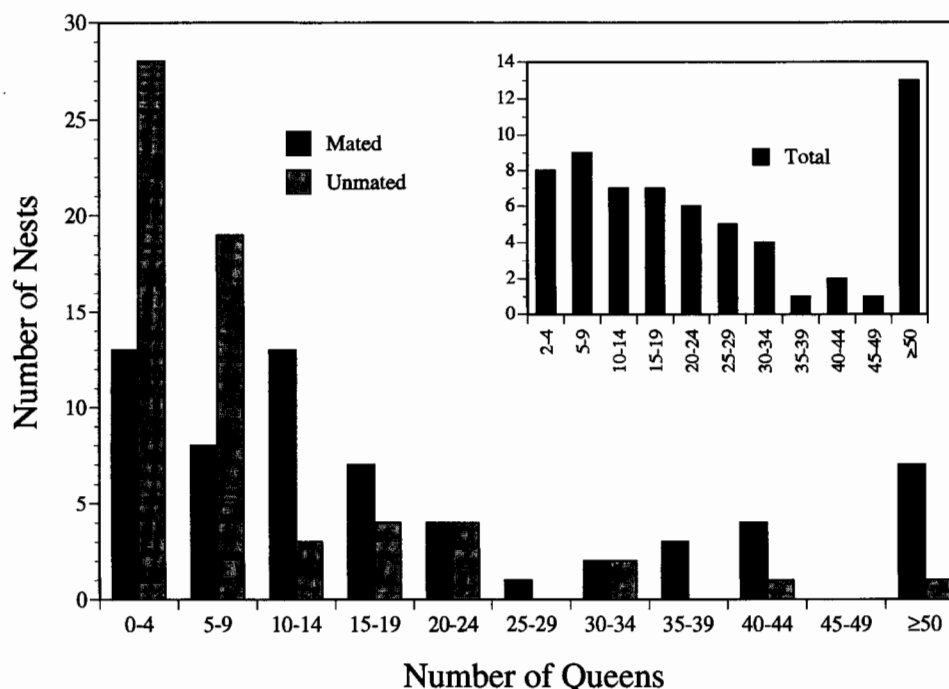
Table 2 shows the mean numbers of nestmate queens and population-wide corrected relatedness values for the total data set. In accord with previous studies (Ross *et al.*, 1996), average queen relatedness within nests was found to be statistically indistinguishable from zero. The per-nest relatedness estimates for mated queens are not significantly correlated with the estimates for unmated queens (Spearman's rank order correlation test,  $r_s = 0.197$ ,  $P = 0.224$ ,  $n = 40$ ), indicating that a relatively high estimate of relatedness among mated queens in a nest does not imply that the estimate for unmated queens will be correspondingly high.

Table 3 gives the results of the tests for a correlation between queen number and queen relatedness in polygyne nests of *S. invicta*. There is no significant association between any relatedness estimate and queen number, indicating that relatedness among nestmate queens does not change systematically as queen number changes. Also, 'population-wide' relatedness was examined for subsets of the population that contained only nests with specified numbers of queens. The results of this analysis are presented in Fig. 2. There is no obvious trend in queen relatedness when cohorts of nests of varying queen number are examined separately, reinforcing the conclusion that relatedness of queens within nests does not correlate with queen number.

It is also important to determine whether variation in relatedness is associated with the number of queens per nest, as would be the case, for instance, if low-queen-number nests comprised two distinct relatedness classes, while high-queen-number nests did not. Nests were assigned to cohorts based on queen number, as above. The standard deviation for the relatedness estimates for each cohort was calculated from a single run of the resampling procedure, and Spearman's rank order correlation coefficient was used to determine the relationship between the standard deviation of the relatedness estimates and mean queen number in each cohort. This procedure was conducted on the data from the first ten runs of the resampling routine. None of these tests revealed a significant relationship between the two variables (data not shown). Thus, there is no evidence that among-nest variation in queen relatedness is associated with queen number.

## Discussion

Significant positive relatedness between individuals within ant colonies is believed to play a pivotal role in relaxing the



**Fig. 1.** Distribution of number of wingless (reproductive) queens in sixty-three confirmed polygyne nests of *Solenopsis invicta* collected in 1990 and 1994.

**Table 1** Relationship between uncorrected relatedness estimates and relatedness estimates corrected for differing numbers of reproductive queens within individual polygyne nests of *Solenopsis invicta*. Values of Spearman's rank order correlation coefficient ( $r_s$ ) are presented along with associated  $P$ -values and sample sizes (numbers of nests, in parentheses) in the top row; bold values indicate significant correlations. The proportions of uncorrected relatedness estimates that were greater than the corresponding corrected relatedness estimates are presented in the middle row. These values were compared with the two-tailed null hypothesis that corrected estimates are greater than or less than uncorrected estimates equally frequently using a normal approximation to the binomial distribution. Values of  $z$  scores indicating a significant departure from this null hypothesis are in bold. Comparisons of the slopes of linear regressions of queen relatedness on queen number obtained using corrected and uncorrected relatedness estimates are shown in the bottom row. The slope obtained using uncorrected relatedness estimates is presented first, with the slope obtained using corrected estimates shown in italics. The probability that the slopes differ significantly from one another is based on a two-tailed test, and the associated  $z$  scores are given.

	Total nestmate queens	Mated nestmate queens	Unmated nestmate queens
Correlation between corrected and uncorrected relatedness estimates	$r_s = \mathbf{0.860}$ $P < 0.001$ (59)	$r_s = \mathbf{0.915}$ $P < 0.001$ (59)	$r_s = \mathbf{0.913}$ $P < 0.001$ (41)
Proportion of uncorrected relatedness estimates greater than corrected estimates	42/58 $z = \mathbf{-3.808}$ $P < 0.001$	43/59 $z = \mathbf{-3.385}$ $P < 0.001$	21/41 $z = 0.000$ $P = 1.000$
Slopes of regression of queen relatedness on number of nestmate queens	$-7.8 \times 10^{-5}$ $-2.4 \times 10^{-4}$ $z = 0.127$ $P = 0.899$	$-1.3 \times 10^{-4}$ $4.2 \times 10^{-4}$ $z = -0.251$ $P = 0.802$	$-5.2 \times 10^{-4}$ $-1.9 \times 10^{-4}$ $z = -0.066$ $P = 0.944$

conditions under which altruism can evolve under kin selection (Hamilton, 1964a,b; Crozier & Pamilo, 1996). Thus, factors that lead to negligible relatedness, such as the presence of numerous unrelated reproductive queens, are potentially problematic. Keller (1995) has formulated a possible proximate explanation for the existence of polygyne ant societies with low nestmate

relatedness. He proposed that the distantly related queens in such colonies originate from within their natal nest, but because the mothers of these queens mated with a male from outside the nest, relatedness among nestmate queens decreases over multiple bouts of queen recruitment. This proposed mechanism of queen adoption should leave a pattern that can be detected empirically;

**Table 2** Mean nestmate queen number and queen relatedness (standardized for differences in queen number) for polygyne nests of *Solenopsis invicta*. Values in parentheses are the 95% confidence limits about the means.

	Total nestmate queens	Mated nestmate queens	Unmated nestmate queens
Number	30.49 (22.70, 38.28)	21.56 (15.77, 27.34)	8.70 (5.54, 11.85)
Relatedness	0.04 (-0.10, 0.17)	0.04 (-0.09, 0.16)	0.02 (-0.11, 0.15)

**Table 3** Correlation between number of nestmate queens and relatedness of these queens in polygyne *Solenopsis invicta*. Spearman's rank order correlation coefficients are presented along with associated *P*-values and sample sizes (numbers of nests, in parentheses).

Total nestmate queens	Mated nestmate queens	Unmated nestmate queens
-0.054	0.048	0.042
<i>P</i> = 0.892	<i>P</i> = 0.719	<i>P</i> = 0.796
(59)	(59)	(41)

queen number should increase and queen relatedness should decrease with the age of a polygyne nest.

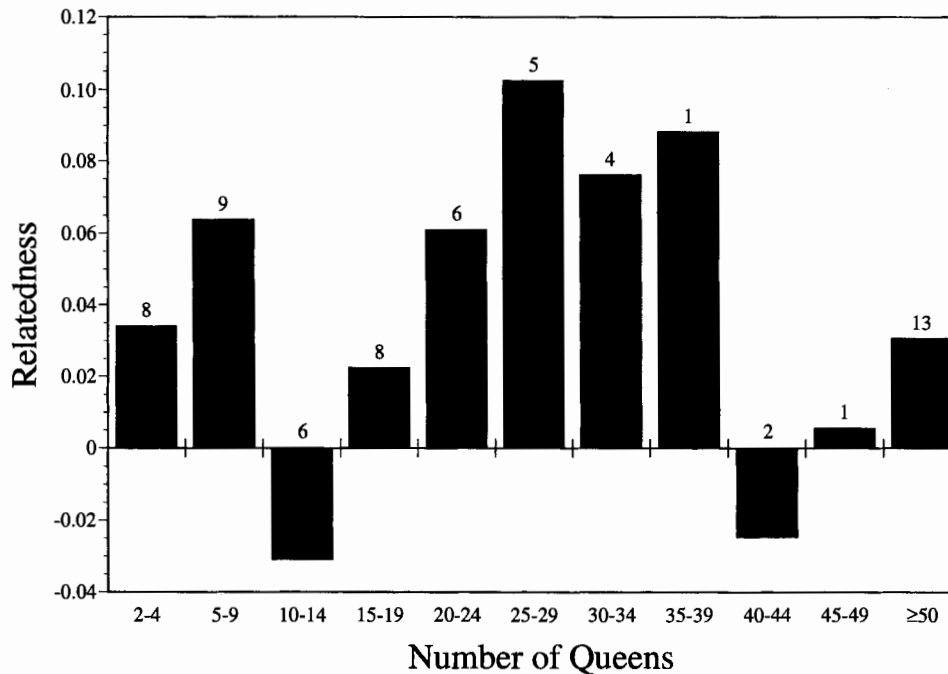
The main finding of this study is that queen relatedness does not decrease predictably with increasing queen number within a single polygyne population of introduced *S. invicta*. This result holds for two large data sets from different years, and for unmated as well as mated queens. The lack of a significant trend in the data suggests that the mode of queen recruitment postulated by Keller (1995) does not invariably occur in introduced *S. invicta*, and thus that polygyne nests of these ants do not behave as closed societies.

These results bear on the manner in which polygyne colonies of *S. invicta* are formed and grow. Monogyny is believed to be ancestral to polygyny in this species, and polygyne populations appear to be derived occasionally from pre-existing monogyny populations (Ross & Keller, 1995). However, the phenotypic and genotypic differences between the two social forms (Ross & Keller, 1995) make it unlikely that transitions between them are simple or that they occur often. Thus, most new polygyne colonies undoubtedly begin as buds of older, pre-existing polygyne colonies (Vargo & Porter, 1989). Because nestmate queens tend to be unrelated, the queens forming a new bud must also be unrelated, barring some tendency for relatives to associate preferentially during the process of budding. This study provides no evidence for such association, because average queen relatedness is close to zero even in the smallest nests (Fig. 2). If small polygyne nests with few queens are relatively young and large nests with many queens are relatively old, the lack of an association between queen number and queen relatedness suggests that polygyne nests of *S. invicta* in North America recruit non-nestmate queens as new reproductives throughout their life cycle. Such acceptance of non-nestmate queens has been documented previously in polygyne nests of this species using mark-recapture techniques (Glancey & Lofgren, 1988; Porter, 1991).

The observed lack of a correlation between queen number

and relatedness is unlikely to result from other causes, such as persistent connections among polygyne nests or the absence of a relationship between queen number and nest age. If nests of polygyne *S. invicta* are often interconnected and exchange of ants is common, as has been suggested (Bhatkar & Vinson, 1987), then the number of queens counted in each nest would not be a particularly good estimate of queen number in the entire colony and a correlation between queen number and relatedness might be obscured. However, there is increasing evidence that polygyne nests are often discrete, definable units among which movement of individuals is restricted. For example, the within-colony genetic variation of workers can be predicted quite well from the number of mated queens present (Ross, 1993). This result is not expected if ants move frequently among nests. Also, mean size of adult workers within nests is strongly negatively correlated with the number of queens present (Goodisman & Ross, 1996). This implies that the number of queens observed at the time of collection has had some significant, long-lasting effect on the within-nest environment, because it takes a worker  $\approx$  30 days to develop from the egg to the adult (O'Neal & Markin, 1975). Frequent movement of queens or workers among nests would be expected to erase the signature of this effect. The other possible explanation for the lack of a correlation between queen number and relatedness, that older nests do not necessarily have higher queen numbers, seems unlikely because polygyne nests, in experimental situations, have been shown to acquire increasing numbers of queens as they age (Vargo & Porter, 1989; Porter, 1991).

If queens that produce workers also produce some sexual progeny, then workers within polygyne *S. invicta* nests are likely to raise a small fraction of sexual brood that are their relatives. Therefore, workers do augment their inclusive fitness through indirect means, even in nests where there are many queens that are virtually unrelated to one another. However, the strength of the indirect fitness component relative to the direct component is likely to be weak, given that average queen relatedness is typically so low. Given the relative weakness of the indirect component, selfish alleles for direct worker reproduction should be selected and spread, and the social system may be expected to break down (Crozier, 1979; Pamilo, 1991). However, there is no indication that polygyne colony functioning or efficiency is being disrupted or that this form is at a significant competitive disadvantage with respect to the monogyny form due to reproductive conflict within the nest (e.g. Greenberg *et al.*, 1992). It is thus important to consider what factors might be acting to maintain polygyne colony cohesion.



**Fig. 2.** Mean relatedness of nestmate queens in polygyne *Solenopsis invicta* for cohorts of nests containing similar numbers of queens. Both mated and unmated queens are included. None of the relatedness values differs significantly from zero (data not shown). Values above columns indicate numbers of nests that fall within each category.

One possible factor maintaining colony integrity is that workers prefer to raise their closest relatives within the colonies (nepotism), thus increasing their inclusive fitness to levels over those predicted on the basis of average nestmate relatedness alone (Carlin *et al.*, 1993; Snyder, 1993). However, workers do not appear to discriminate among more or less closely related sexuals in relevant contexts in laboratory assays (Ross, 1988; DeHeer & Ross, 1997). A second possibility is that there is a high degree of reproductive skew among queens, and that the relatedness of sexuals to the workers that raise them is higher than implied from the low relatedness of nestmate queens. Substantial reproductive skew has been observed in polygyne nests of *S. invicta* in the laboratory, particularly with respect to sexual production (Ross, 1988), but the actual relatedness measured between workers and sexual brood in the field is indistinguishable from zero (Ross *et al.*, 1996), indicating that the queens whose progeny are overrepresented among the sexuals do not have their progeny overrepresented among the workers.

Colony-level selection has also been cited as a potentially important process that may lead to the success of polygyne colonies containing unrelated queens. For example, such polygyne ants are frequently found in disturbed habitats, which has been viewed as support for the hypothesis that they may be particularly successful in some environments (Hölldobler & Wilson, 1977; Nonacs, 1993; Passera, 1994). However, such ecological explanations are unwarranted in *S. invicta*, because monogyne and polygyne colonies appear to occupy identical habitats (Porter *et al.*, 1991), thus casting doubt on the hypothesis that polygyne colonies have a competitive advantage

in certain areas. Another explanation holds that the increase in within-colony genetic variation provided by multiple unrelated reproductives can be of selective importance in protecting the colony against attacks by parasites and diseases (Sherman *et al.*, 1988; Shykoff & Schmid-Hempel, 1991; Keller & Reeve, 1994). However, introduced populations of *S. invicta* are largely free of the parasites and diseases found in native populations (Orr *et al.*, 1995), so it is unlikely that high within-colony genetic variation is important in this context in their present environment.

Finally, it is possible that the inability of *S. invicta* workers to lay eggs (Hölldobler & Wilson, 1990) plays a role in the maintenance of these polygyne societies. Supporting this view is the fact that other polygyne ants with low queen relatedness frequently have sterile workers (Bourke & Franks, 1995; Keller, 1995). In introduced *S. invicta*, workers may have little choice but to reap the very low inclusive fitness benefits that may be available when only a small fraction of the sexual brood they tend is composed of relatives (Keller, 1995).

The postulated acceptance of non-nestmates as new reproductives reflects a significant change in life-history strategy for *S. invicta* (Ross *et al.*, 1996), which may reflect differences in selection pressures between the native and newly colonized environments. North America has provided a generally permissive environment for fire ants, as they are afflicted by far fewer natural enemies than in their native range in South America (Orr *et al.*, 1995). This has led to an increased density of *S. invicta* nests, potentially leading to saturation of available nesting sites and a decrease in the success of queens attempting to found colonies independently. Under

these circumstances, prereproductive queens may be under strong selection to enter established nests (Herbers, 1986, 1993; Nonacs, 1988; Pamilo, 1991; Ross & Keller, 1995), leading to the frequent adoption of foreign queens by polygyne nests and observed low queen relatedness.

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