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Research article

Multiple mating in the ant *Cataglyphis cursor:* testing the sperm limitation and the diploid male load hypotheses

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Abstract. Multiple mating (*i.e.*, polyandry) by queens in social Hymenoptera is expected to weaken social cohesion since it lowers within-colony relatedness, and hence, indirect fitness benefits from kin selection. Yet, there are many species where queens mate multiply. Several hypotheses have been put forward to explain the evolution and maintenance of polyandry. Here, we investigated the 'sperm limitation' and the 'diploid male load' hypotheses in the ant *Cataglyphis cursor.* Genetic analyses of mother-offspring combinations showed that queens mate with up to 8 males, with an effective mating frequency of 3.79. Significant paternity skew (unequal contribution of the fathers) was detected in 1 out of 5 colonies. The amount of sperm stored in the spermatheca was not correlated with the queen mating frequency, and males carry on average enough sperm in their seminal vesicles to fill one queen's spermatheca. Analyses of the nuclear DNA-content of males also revealed that all were haploid. These results suggest that the 'sperm limitation' and the 'diploid male load' hypotheses are unlikely to account for the queen mating frequency reported in this ant. In light of our results and the life-history traits of *C. cursor*, we discuss alternative hypotheses to account for the adaptive significance of multiple mating by queens in this species.

Keywords: Ants, flow cytometry, microsatellites, polyandry, sperm.

Introduction

A key challenge in evolutionary biology is to understand the adaptive significance of multiple mating by females (polyandry). Mating is generally assumed to be associated with costs to females in terms of energy expenditure, exposure to predation, and sexually transmitted parasites and pathogens (Daly, 1978; Chapman et al., 1995, 2003). Yet, females of many animal species mate with several males (Birkhead and Moller, 1998; Arnqvist and Nilsson, 2000; Eberhard, 1996). Social Hymenoptera are no exception to this rule. Obligate multiple mating by queens has evolved repeatedly in bees, wasps and ants (Crozier and Pamilo, 1996; Crozier and Fjerdingstad, 2001; Brown and Schmid-Hempel, 2003; Boomsma et al., 2005). For instance in ants, where effective queen mating frequency ($M_{e,p}$) is usually lower than 2 (Boomsma and Ratnieks, 1996; Strassmann, 2001; Crozier and Fjerdingstad, 2001), high polyandry levels have been reported in the genera *Atta* ($M_{e,p}$= 3.1, Murakami et al., 2000), *Cardiocondyla* ($M_{e,p}$= 3.3, Lenoir et al 2007), *Acromyrmex* ($M_{e,p}$= 3.9, Boomsma et al. 1999), *Pogonomyrmex* ($M_{e,p}$= 6.8, Cole and Wiernasz, 2000), *Neivamyrmex* ($M_{e,p}$= 12.8, Kronauer et al., 2007), *Eciton* ($M_{e,p}$= 12.9, Kronauer et al., 2006), *Dorylus* ($M_{e,p}$= 17.5, Kronauer et al., 2004), and *Aenictus* ($M_{e,p}$= 18.8, Kronauer et al., 2007).

Several genetic and non-genetic benefits have been proposed to explain the evolution and maintenance of multiple mating in social Hymenoptera. A first set of hypotheses stresses the benefits of increased genetic diversity in the offspring, e.g., by enhancing colony resistance to parasites and pathogens (Hamilton, 1987; Sherman et al., 1988), raising the efficiency of the colony

and its overall productivity through a more efficient division of labor among workers (Crozier and Page, 1985; Robinson and Page, 1995; Mattila and Seeley, 2007), or reducing worker-queen conflict over the sex ratio and male parentage (Trivers and Hare, 1976; Moritz, 1985; Ratnieks, 1988; Ratnieks and Boomsma, 1995; Sundström and Ratnieks, 1998). Empirical tests of these hypotheses have yielded support in some species but not in others (reviewed in Crozier and Fjerdingstad, 2001; Brown and Schmid-Hempel, 2003; Boomsma et al., 2005).

One proposal for the evolution of multiple mating directly connected to the advantages of genetic diversity is that polyandry would have been selected for to reduce the variance in the production of diploid males ('diploid male load' hypothesis, Crozier and Page, 1985; Pamilo et al., 1994). Hymenoptera are characterized by haplodiploid sex determination, whereby the composition of alleles at a single locus is the primary signal of sex determination (single-locus complementary sex determination or sl-CSD; Whiting, 1943; Cook and Crozier, 1995). Diploid individuals heterozygous at the sex locus develop into females, whereas haploid individuals hemizygous at the sex locus develop into males. Diploid males arise when there is homozygosity at the sex locus, that is, when both parents transmit identical alleles at the locus to the offpring (*i.e.,* matched mating; Cook and Crozier, 1995). Production of diploid males potentially imposes a high cost on colony survival, because queens mated with a single male carrying the same sex allele will produce 50 % diploid males instead of workers, which may be detrimental for colony foundation (Ross and Fletcher, 1986; Duchateau and Mariën, 1995). Furthermore, diploid males constitute particularly high fitness costs to the colony since they are usually sterile or they father sterile, triploid female progeny (Krieger et al., 1999; Liebert et al., 2004; de Boer et al., 2007; but see Cowan and Stahlhut, 2004). Therefore, multiple mating also reduces the costs associated with mating with diploid males. In the honeybee, workers remove diploid males before maturation (Woyke, 1963, 1980; Ratnieks, 1990; Santomauro et al., 2004), which greatly reduces the probability of young queens mating with such males. By contrast, there is no direct empirical evidence for the selective removal of diploid male brood in wasps, bumble bees, or ants, where adult diploid males are indeed produced in several species (e.g., wasps: Tsuchida et al., 2002; Liebert et al., 2004; bumble bees: Ayabe et al., 2004; ants: Pamilo et al., 1994; Ross and Fletcher, 1986; Krieger et al., 1999; Yamauchi et al., 2001).

The second set of hypotheses suggests that females may derive direct benefits from mating multiply, e.g. by receiving a sufficient sperm reserve. Ant queens usually mate on a single nuptial flight (Hölldobler and Wilson, 1990). The amount of sperm received during the limited time window of mating flight will directly affect the queen's lifetime output of fertilised eggs, and hence, the number of workers and female sexuals produced. Therefore, multiple mating by queens during the nuptial flight may have been selected for to achieve a greater supply of sperm to maintain large and long-lived colonies ('sperm-limitation' hypothesis, Cole, 1983). To date, the 'sperm-limitation' hypothesis remains poorly studied. In the leaf-cutting ant *Atta colombica*, which constitutes huge colonies of several thousands of workers, multiple-mating effectively increases the queen's sperm store (Fjerdingstad and Boomsma, 1998). Similarly, the sperm content of queens' spermatheca increases almost linearly with queen mating frequency in *Formica aquilonia* (Fortelius, 2005). Indirect support for this hypothesis also comes from the positive association between paternity rate and colony size for monogynous (single-queen) ants (Boomsma and Ratnieks, 1996), and from increased queen mating frequency with decreasing sperm supply of drones in honeybees (Kraus et al., 2004).

In this study, we investigated the 'sperm limitation' and the 'diploid male load' hypotheses as possible causes for the evolution of polyandry in the ant *Cataglyphis cursor.* Colonies are headed by single, multiple-mated queens showing natural variations in their mating frequency (Pearcy et al., 2004a). They are relatively small, never exceeding 2800 workers per colony (Pearcy and Aron, 2006). A remarkable feature of this species is that queens use alternate modes of reproduction for the production of reproductive and non-reproductive daughters. Workers are produced by sexual reproduction from fertilized eggs, while new queens are almost exclusively produced by automictic thelytokous parthenogenesis with central fusion of the polar nuclei (Pearcy et al., 2006). This mode of parthenogenesis augments the rate of homozygosity of reproductive daughters, and hence, the probability of homozygosity at the sex determining locus. Consequently, production of sterile diploid males in *C. cursor* may stem from two different sources : matched mating, resulting in fertilized eggs developing into diploid males rather than workers and, more importantly, from thelytokous parthenogenesis resulting in production of diploid males rather than queens (Fig. 1).

We first determined the queen-mating frequency and the level of skewness in paternity by genetic analyses of mother-offspring combinations. From this data, we tested for a possible relationship between the absolute queen mating frequency and colony size. Second, we compared the sperm content between the queen spermatheca and the males' seminal vesicles, to determine whether the amount of sperm cells stored in the spermatheca was associated with the number of mates per queen. Finally, we estimated the proportion of adult diploid males reared in the study population.

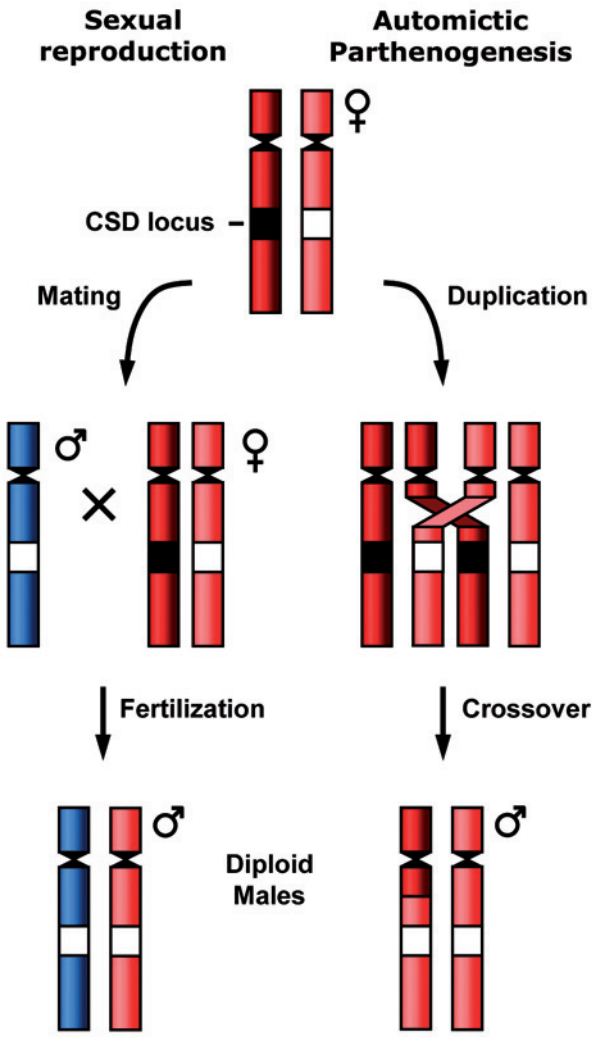

Figure 1. In Hymenoptera, diploid males arise when a diploid individual carries two identical alleles at the complementary sex determining (CSD) locus. In *C. cursor*, both sexual and asexual reproduction may potentially induce homozygosity at the CSD-locus, when the queen mates with a male carrying one shared allele or when heterozygosity at the CSD-locus is lost through automictic parthenogenesis.

Material and methods

Field collection and sampling

Twenty-three colonies of *Cataglyphis cursor* were excavated at the end of April/early May, before the emergence of the first sexuals, at St-Hyppolite (southern France; 42.82° North, 2.99° East) between 2001 and 2004 (see Pearcy et al. (2004a) and Pearcy and Aron (2006) for details). All adults (queens and workers) as well as brood at various stages (eggs, larvae, and sexual pupae) were collected and brought into the laboratory. The number of adult workers per colony was counted for N= 12 colonies (mean colony size ± SD= 832.1 ± 610.1; range: 123–2495). Colonies were maintained under laboratory conditions (26 ± 2°C; 12 h:12 h L:D) and fed on cockroaches, sugar water, and grapes. They were censused twice a week and all sexuals emerging from the pupae were collected; males were kept apart with a sample of workers for subsequent sperm and ploidy level analyses (see below), whereas young sexual females were deep-frozen for subsequent genetic analyses.

Queen mating frequency and paternity skew

New queens of *C. cursor* are produced parthenogenetically (Pearcy et al., 2004b), and both queen turnover and colony fission events can result in the coexistence of workers with different yet genetically similar mother queens. Therefore, determining the number of worker patrilines in a colony on the basis of individuals collected in the field (e.g., Fournier et al., 2008) greatly increases the risk of overestimating the actual queen mating frequency. The number of matings per queen was thus estimated from mother-offspring combinations genetic analyses. For this purpose, all the brood was carefully removed from the 23 laboratory nests after the production of sexuals. After 4 months, the queen and the callow workers produced in each nest were taken for subsequent genetic analyses. Since development from the egg to the adult stage lasts up to 40 days, all callows originated from eggs laid by the colony queen in the laboratory.

A sample of 673 callow workers (mean ± SD= 28.0 ± 11.5; n= 23) and their mother queen were genotyped at four polymorphic microsatellite loci (*Ccur11*, *Ccur46*, *Ccur58*, and *Ccur63b*; Pearcy et al., 2004b). Individual ant DNA was extracted by homogenization in a digestive solution (100mM NaCl, 50mM Tris, 1mM EDTA, 0.5 % SDS, and 200 µg/ml proteinase K (BIOGENE)) and incubated for 2 hours at 55°C. Genomic DNA was purified by phenol/chloroform and precipitated with ethanol following standard protocols (Sambrock et al., 1989), and then resuspended in 100 µl. Amplifications were carried out in a 10 µl volume using the standard 10x Buffer and Taq from the QIAGEN Polymerase kit (Pearcy et al., 2004a). Amplified fluorescent fragments were visualized using an automated ABI Prism 3100 sequencer.

The absolute number of matings per queen (M_p) was determined from pedigree analysis of mother-offspring combinations. This is straightforward due to the haploidy of males since, for each locus, a male gives the same allele to all his offspring. The number of distinct male genotypes inferred per colony provides the minimal number of mates of each queen. The effective number of matings per queen ($M_{e,p}$) was calculated following Nielsen et al. (2003; eq. 16),

$$M_{e,p} = \frac{(n-1)^2}{\sum_{i=1}^{k} p_i^2 (n+1)(n-2) + 3 - n}$$

where n is the sample size and p_i is the proportional contribution to the brood of the ith mate.

Because two males could share the same alleles at the 4 loci studied, we estimated this non-detection error for each colony by calculating the probability that two mates bear the same alleles according to Boomsma and Ratnieks (1996),

$$P_{non\text{-}detect} = \sum_j \prod_i f_{ij}$$

where f_{ij} is the frequency at the population level of the allele carried by the jth male at the ith locus. On the other hand, there is always the possibility that an additional patriline was not sampled but still present among the brood, because of the limited sample size. This non-sampling error was estimated following Foster et al. (1999),

$$P_{nonsampling} = (1\text{-}f)^n$$

where n is the number of offspring analyzed and f is the proportional representation of a father among the brood.

Skewness in paternity (the unequal contribution of each father to the brood) of a given colony was quantified according to Pamilo and Crozier (1996),

$$S = \frac{M_p - M_{e.p}}{M_p - 1}$$

where M_p is the total number of male mates and $M_{e,p}$ is the effective number of male mates, both estimated from the mother-offspring pedigrees. For each colony, we determined the statistical significance of the male skew using a *G*-test for goodness-of-fit under the null hypothesis that all males contributed equally to progeny production. Because unequal paternity could arise from sampling effect even when the fathers have equal numbers of offspring (Foster and Ratnieks, 2001), we also compared the paternity skew in each sample with the skew from 10000 random samples taken from a virtual colony with equal father contribution. A sample was considered skewed when *S* was greater than at least 95 % of the simulated unskewed samples.

Sperm count

Estimating the effect of multiple mating on sperm storage was performed on a sub-sample of n= 11 colonies for which the queen mating frequency was previously determined. The queens were dissected and the number of sperm stored in their spermatheca was counted. The age of queens from field colonies was unknown and can represent a source of variation in our data because we expect sperm storage to decrease with age. However, because *C. cursor* queens have a short life expectancy and queen productivity is low (Pearcy et al., 2006), the influence should be limited. The seminal vesicles of a sample of n= 33 mature males at the time of nuptial flight (*i.e.* one or two weeks after their emergence) were also dissected and the sperm content was counted. Testes were always found degenerated (as compared to newly-emerged males), which confirms that males were mature at the time of dissection. We averaged sperm content whenever males originated from the same colony, to avoid a potential bias in the data. Queens and males were dissected in Ringer solution. Both spermathecae and seminal vesicles were emptied in a staining solution of DAPI fluorochrome (4', 6-diamidino-2-phenylindole). The number of sperm was then counted by flow cytometry (Ploidy Analyser PAI, Partec), a method that gives reliable and repeatable counts of sperm samples (Aron et al., 2003; Cournault and Aron, 2008).

Diploid males

To test for the production of adult diploid males, we sampled n= 179 males from N= 8 colonies. The potential homozygosity excess in the queens' parthenogenetic lineages was estimated for these colonies through *F*-statistics using the program Relatedness 4.2c (Queller and Goodnight, 1989). The ploidy level (haploid or diploid) of males was determined by using only cells from the head rather than from the whole body, because haploid Hymenopteran males have diploid muscle cells (Aron et al., 2005). DNA of cell nuclei was stained with DAPI fluorochrome, and the nuclear DNA-content of each male's cells was determined by flow cytometry.

Results

Queen mating frequency and paternity skew

The distribution of genotypes in parent-offspring combinations was consistent with queens being multiply-mated. In the 23 colonies sampled, we found that queens were mated with 2 to 8 different males. The harmonic mean number (± SD) of fathers detected per colony was M_p= 4.91 ± 1. 40, and the average effective number of matings per queen was $M_{e,p}$= 3.79 ± 1.48. Both the population-wide non-detection error due to two males bearing the same alleles at all loci ($P_{non\text{-}detect}$= 0.003 ± 0.003) and the non-sampling error due to limited sample sizes ($P_{non\text{-}sampling}$= 0.073 ± 0.049) were very low. It is therefore unlikely that our data were affected by these potential sources of errors. The absolute queen mating frequency (M_p) was not significantly associated with colony size at the time of colony collection (Spearman rank correlation, r_s= 0.29, n= 12, P= 0.35).

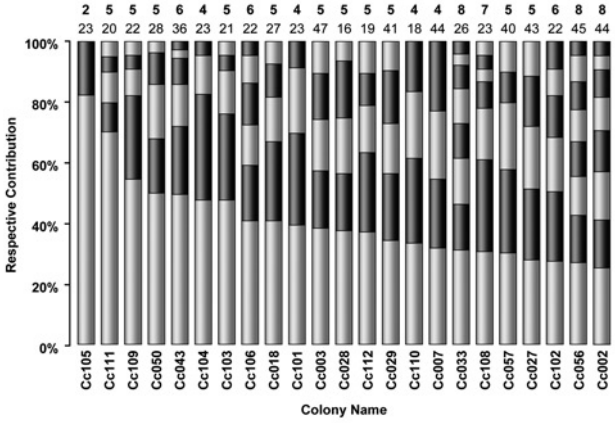

Figure 2. The frequency distributions of patrilines (offspring sired by different males) as estimated from parent-offspring combinations. Patrilines are shown by alternate shading patterns, with the total number of matings (M_p) and the sample size indicated above the bars for each colony. Colonies were ordered by decreasing proportion of the largest patriline.

Pedigree analysis showed that the respective contribution of the male mates varied across colonies, with the majority male siring 0.25 to 0.83 of the offspring (Fig. 2). The paternity skew over all colonies ranged from 0.03 to 0.74 and was on average S ± SD = 0.22 ± 0.23. We detected a significant deviation from equal father contribution in 10 colonies, but these deviations remained significant only in 5 colonies (22 %) after Bonferroni correction (*G*-test for goodness-of-fit, P= 0.003, P= 0.001 (twice), and P< 0.001 (twice)). Our simulations confirmed that paternity skew was significantly higher than expected from sampling effect in those 5 colonies (also corrected for repeated analyses). In the remaining colonies, there was no evidence for unequal contribution of fathers.

Sperm count

For the 11 *C. cursor* queens analysed, the mean spermathecal content ranged from 37.8 $\times 10^3$ to 165.8 $\times 10^3$ sperm and was on average n= 109.7 ± 37.9 $\times 10^3$ sperm. This value was lower, but not significantly so, than the sperm number found in male seminal vesicles, which ranged from 76.9 $\times 10^3$ to 800.6 $\times 10^3$ with an overall mean of 235.1 ± 254.9 $\times 10^3$ sperm (Student *t*-test, P= 0.18).

In this sample, the average queen had mated with M_p = 4.4 males (SD: 1.26, range: 2 to 7). The relationship between the number of sperm stored per queen and the

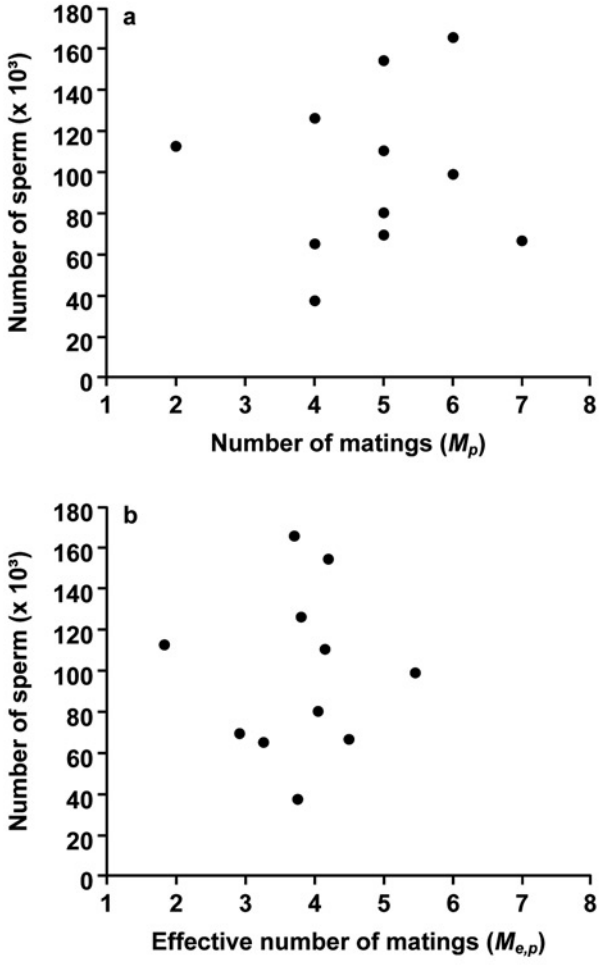

Figure 3. Number of sperm stored per *Cataglyphis cursor* queen as a function of the absolute number of mates M_p (a), and effective number of mates $M_{e,p}$ (b).

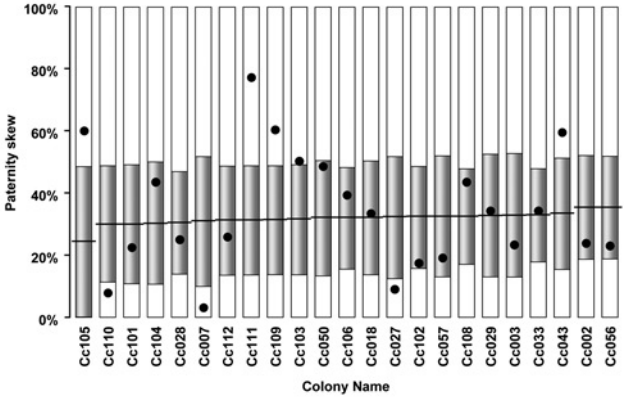

Figure 4. Observed paternity skew (black dots) and expected level of skew stemming from differential male sperm contribution (grey boxes) for each colony. The boxes represent average (line) ± standard deviation for simulation, with the number of matings (M_p) and sample size as parameters for each colony. Colonies were ordered by increasing average skew values.

number of mates (M_p) was not significant (r_s = 0.11, n = 11, P = 0.87; Fig. 3a). There was also no correlation between the number of sperm stored and the effective mating frequency ($M_{e,p}$) of queens (r_s = -0.08, n = 11, P = 0.82; Fig. 3b). The very low slope of the least square regression suggests that queens acquire most of the sperm during one mating (> 90 ×10³ sperm, on average), while each additional mating merely adds 1.6 ×10³ sperm, on average. There was no association between the detected number of mates and the sample size (r_s = –0.10, n = 11, P = 0.77), indicating that the slight variation in the sample size had no effect on our estimates of the queen mating frequency.

To test whether the variability in sperm content of male seminal vesicles may explain the observed paternity skew among workers, we estimated the expected skew for each colony under the assumptions that males deliver their full package and that the probability of a male fathering a given offspring is equal to its sperm contribution in the queen spermatheca. Our simulations showed that the observed paternity skew falls within the range of the expected skew resulting from different sperm contribution in 15 colonies out of 23 (Fig. 4).

Diploid males

As expected from the parthenogenetic production of new queens (Pearcy et al., 2004a), there was a significant excess of homozygosity in queens and their reproductive daughters (F_{is} = 0.218 ± 0.088, P= 0.008) but not in workers (F_{is} = 0.034 ± 0.041, P= 0.20), in the 8 colonies sampled for diploid male production. Despite the high level of inbreeding in reproductive females, flow cytometric analyses showed that none of the 179 males sampled were diploid.

Discussion

Our data show that queens of *Cataglyphis cursor* are strictly polyandrous, mating with up to 8 males. Consistent with these results, field observations indicate that queens repeatedly leave the mother nest to mate with the surrounding males, then re-enter the nest (Lenoir et al., 1988; pers. obs.). The fact that queens actively seek to mate several times strongly suggests that polyandry represents a valuable increase in their fitness. However, our results support neither the 'sperm limitation' hypothesis, nor the 'diploid male load' hypothesis as the evolutionary causes for polyandry in this species. Overall, we found no association between colony size and mating frequency of the queen. The quantity of sperm carried by males was highly variable, and certain males carry relatively few sperm. However, the average (= 110 ×10³ sperm) or the maximum (= 166 ×10³ sperm) quantity of sperm stored in one queen spermatheca can be reached with a probability higher than 0.99 with 2 or 3 matings,

respectively. The variation in the quantity of sperm carried by *C. cursor* males can therefore not account for up to 8 matings detected in this study. Furthermore, our analyses show that the number of sperm stored by each queen does not increase with additional mating. Two non-mutually-exclusive hypotheses may explain this result. First, the size of the spermatheca may limit the amount of sperm stored. Second, if males can detect whether females are already mated, they might restrain the amount of sperm transferred to save some sperm for a potential subsequent mating (Boomsma, 1996; Boomsma et al., 2005). Whatever hypothesis prevails, both suggest that remating is not a strategy for queens to acquire more sperm.

Across ants, Boomsma and Ratnieks (1996) showed that the number of matings is associated with colony size when polygynous species are excluded from the analysis, as predicted by the 'sperm-limitation' hypothesis (Cole, 1983). In *Atta colombica,* multiple mating allows queens to effectively increase their sperm store, suggesting that polyandry could be an adaptive strategy to avoid sperm depletion (Fjerdingstad and Boomsma, 1998). It should be noted, however, that colonies of this leaf-cutter ant may contain several millions of workers and that queens are long-lived (10 to 16 years). Large colony size is also typical of other polyandrous species such as *Acromyrmex octospinosus* (up to 15000 workers; Dijkstra and Boomsma, in press), *Pogonomyrmex occidentalis* (up to 8800 workers; Lavigne, 1969), *Neivamyrmex nigrescens* and *Aenictus laeviceps* (ca. 100 000 workers, Schneirla, 1971), *Dorylus molestus* (millions of workers; Raignier and van Boven, 1955) and *Eciton burchellii* (up to half a million workers; Franks, 1985 – but see Kronauer and Boomsma, 2007 for arguments against the sperm limitation hypothesis in army ants). By contrast, in *C. cursor*, colonies are quite small, usually comprising hundreds of workers (range: 78–2658; N= 57 colonies; Pearcy and Aron, 2006), and queens have a short life expectancy (Pearcy et al., 2006). Overall, these results suggest that, in this species, sperm amount is not a limiting factor for queen fitness and that multiple mating is not selected for to avoid sperm depletion.

In species such as *C. cursor* where new colonies are produced by fission, a huge amount of resources is invested in each young queen. Under these circumstances, polyandry should be particularly adaptive because it considerably reduces the proportion of young queens with high (and potentially lethal) levels of diploid male load (Kronauer et al., 2007). Importantly, in this species diploid males can arise either from sexual or asexual reproduction by the queens. Production of diploid males through sexual reproduction is expected to be negligible, because queens and her mates are unrelated (Pearcy et al., 2004a). By contrast, automictic parthenogenesis greatly increases inbreeding (Pearcy et al., 2006). Consistent with these expectations, our data show a significant excess of homozygosity in queen lineages, but not in workers. Thus, in *C. cursor*, polyandry should not significantly influence the production of diploid males; rather, diploid males (if any) are expected to be primarily parthenogenetically-produced. However, polyandry could still be selected for by the queens to lower the variance of the fitness costs associated with the risk of mating with a sterile diploid male. Interestingly, not a single mature diploid male was found in the study population, indicating that such males are rare or even absent. Two proximate mechanisms may explain the absence of adult diploid males. First, genetic mechanisms may prevent homozygosity at the CSD loci, such as multiple-loci complementary sex determination (ml-CSD) (Crozier, 1971) or genomic imprinting (Beukeboom, 1995). To date, ml-CSD has not been directly shown in a social Hymenoptera, but good evidence against single-locus complementary sex determination has recently been published for *Cardiocondyla elegans* (Schrempf et al., 2006). Genomic imprinting cannot be tested without appropriate cytogenetic markers, such as the paternal sex-ratio chromosome (PSR) in *Nasonia vitripennis* (Dobson and Tanouye, 1998; Beukeboom and Werren, 2000), which are not available for any ant species. The proximity of the CSD locus with the centromere of the chromosome could be another genetic mechanism to account for the lack of diploid males in species with parthenogenesis-driven homozygosity. Automictic parthenogenesis increases homozygosity for loci where recombination occurs during meiosis. If the CSD locus is located near a chromosomal centromere, where very few recombination events occur, one should expect few transitions to homozygosity for this locus. The second proximate mechanism accounting for the absence of diploid males in *C. cursor* is that such males are produced but selectively eliminated before pupation by workers. Whatever mechanism is involved, the complete absence of mature diploid males in our sample suggests that polyandry was not selected for by the queens to circumvent the costs of mating with sterile diploid males. So far, the influence of the 'diploid male load' on queen mating frequency in social insects has not been demonstrated. Mathematical models indicate that, in the honeybee *Apis mellifera*, low mating frequency would increase the risks of high mortality among the brood due to diploid males (Tarpy and Page, 2001). However, the authors admitted that diploid load could hardly account for more than 10 mating, which often occurs in this species.

This work, together with the unusual life history of *C. cursor*, allows further consideration of the possible evolutionary causes of multiple mating in this ant species. If multiple mating by queens increased their fitness through post-copulatory sperm competition, one would expect a significant bias in male contribution to the brood, with the fittest male(s) fathering a larger fraction of workers ('polyandry for sperm competition' hypothesis, Parker, 1970; Simmons, 2001). Our data show a significant unequal contribution of fathers in only 5 colonies out of 23 (22%). That sperm competition – if any – has no obvious effect in most colonies gives weak support for the

hypothesis that polyandry effectively increases queen fitness through sperm competition. However, one may not completely exclude this hypothesis since our experiments were not designed to test the existence of potential competition between the sperm of different fathers. Actually, the high variance in the sperm content found in male seminal vesicles alone may explain most of the paternity bias detected in the colonies, as suggested by our simulations. According to the 'mating by convenience' hypothesis (Thornhill and Alcock, 1983), sexual coercion was also evoked as a cause of multiple mating. The sexual coercion hypothesis is unlikely in *C. cursor*, where new queens leave the nest repeatedly to copulate with several males close to the nest entrance (Lenoir et al., 1988; pers. obs.). Rather, multiple mating is an active strategy by queens and not a mere consequence of male willingness to mate in this species. Genetic variability acquired through multiple mating has been assumed to enhance colony task efficiency (Crozier and Page, 1985; Robinson and Page, 1995; Mattila and Seeley, 2007). However, a recent test of this hypothesis in *C. cursor* showed that increased genetic diversity within colonies does not result in more polymorphic workers, and task performance is not correlated with patriline (Fournier et al., 2008). Within-colony genetic diversity has also been shown to effectively lower the parasite pressure in social insects (Hughes and Boomsma, 2004; Baer and Schmid-Hempel, 1999). That parasite pressure favours multiple mating by queens may prove particularly relevant for a scavenger ant such as *C. cursor*, where workers are potentially exposed to pathogens developing on dead arthropods. Queens might also exploit multiple mating to reduce the conflict that opposes them to the workers regarding the maternity of males (Ratnieks, 1988). Workers of *C. cursor* have retained ovaries and can produce males through arrhenotokous parthenogenesis and females through thelytokous parthenogenesis (Cagniant, 1980). Polyandry lowers within-colony relatedness and results in that workers are more related to their reproductive sisters and brothers ($r = 0.62$ and $r = 0.32$, respectively; Pearcy and Aron, 2006) than to the parthenogenetic daughters and sons produced by their worker nestmates ($r = 0.42$ and $r = 0.21$, respectively; estimated from within-colony relatedness). Multiple mating may therefore have been selected for by queens to force workers to rear their sibs instead of their own offspring.

In conclusion, our data show that neither the 'sperm-limitation' hypothesis nor the 'diploid male load' hypothesis can account for multiple mating in the ant *Cataglyphis cursor.* Moreover, we did not detect the presumed effects of post-copulatory sperm competition, and the observations of female mating behaviour are direct evidence against the 'mating by convenience' hypothesis. Interestingly, two biological traits of *C. cursor* considerably reduce the costs of mating multiply. First, the absence of a nuptial flight lowers the energetic costs and the risks of predation associated with multiple mating (Boomsma and Ratnieks, 1996). Second, in this species, reproductives of both sexes are produced asexually, so that multiple mating does not affect worker inclusive fitness (Pearcy and Aron, 2006). In light of our results and the natural history traits of this species, future work should focus on tests of the 'genetic diversity' and the 'conflict' hypotheses, as relevant explanations to account for the evolution of polyandry in this species.

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