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ORIGINAL ARTICLE

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Dual mechanism of queen influence over sex ratio in the ant *Pheidole pallidula*

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Abstract Social Hymenoptera are general models for the study of parent-offspring conflict over sex ratio, because queens and workers frequently have different reproductive optima. The ant *Pheidole pallidula* shows a split distribution of sex ratios with most of the colonies producing reproductives of a single sex. Sex ratio specialization is tightly associated with the breeding system, with single-queen (monogynous) colonies producing male-biased brood and multiple-queen (polygynous) colonies female-biased brood. Here, we show that this sex specialization is primarily determined by the queen's influence over colony sex ratio. Queens from monogynous colonies produce a significantly more male-biased primary sex ratio than queens from polygynous colonies. Moreover, queens from monogynous colonies produce a significantly lower proportion of diploid eggs that develop into queens and this is associated with lower rate of juvenile hormone (JH) production compared to queens from polygynous colonies. These results indicate that queens regulate colony sex ratio in two complementary ways: by determining the proportion of female eggs laid and by hormonally biasing the development of female eggs into either a worker or reproductive form. This is the first time that such a dual system of queen influence over colony sex ratio is identified in an ant.

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Introduction

Studies of queen-worker conflicts over sex ratio in social Hymenoptera have emerged as prime tests of kin selection and inclusive fitness theories (Hamilton 1964a; Hamilton 1964b). The haplodiploid sex-determining system of Hymenoptera (males develop from unfertilized-haploid eggs, females from fertilized-diploid eggs) produces asymmetries in genetic relatedness among colony members, with workers being more closely related to females than to males. For instance, in a colony headed by a single once-mated queen, workers are three times more related to sisters ('life-for-life relatedness': r=0.75) than to brothers (r=0.25). Workers, who reproduce indirectly by rearing the queen's offspring, therefore increase their inclusive fitness by producing a 3:1 female-biased sex ratio among sexual offspring (Trivers and Hare 1976). Queens, however, are equally related to daughters and sons so that natural selection should act on queens to favor an equal investment in male and female sexuals at the population level (Trivers and Hare 1976; Bourke and Franks 1995; Crozier and Pamilo 1996; Beekman and Ratnieks 2003). Although all sex ratios at the level of the colony are equally fit in large populations at equilibrium (Kolman 1960), queen-worker conflict should occur in every colony, leading queens in all colonies to produce more males than workers would prefer. The resulting conflict has likely promoted the evolution of behavioral and physiological mechanisms that provide both the workers and queens some control over sex ratio.

Although recent studies have elucidated some of the control mechanisms employed by ant workers, the regulation of brood sex ratio by queens is still poorly understood. Intraspecific studies show that workers bias sex ratios adaptively by rearing mostly females in colonies with high relatedness asymmetry (e.g. colonies headed by one or

several unrelated, singly-mated queen(s)) and by producing mostly males in colonies with lower relatedness asymmetry (e.g. those with multiply-mated queens or several related queens) (Queller and Strassmann 1998; Chapuisat and Keller 1999). In ants, workers have been shown to bias sex allocation by destroying male larvae (*Linepithema humile*, Aron et al. 1994, 1995; Passera and Aron 1996; *Formica exsecta*, Sundström et al. 1996; Chapuisat et al. 1997; *Plagiolepis pygmaea*, Aron et al. 2004). In some species, workers also seem able to channel a larger proportion of the female brood into the queen caste rather than the worker caste through differential feeding (*Leptothorax acervorum*, Hammond et al. 2002).

Similar control mechanisms for queens have not been identified, but there is indirect evidence of their existence. Sex investment ratio frequently is less female-biased than the worker optimum, and even close to the queen equilibrium in some species (Bourke and Franks 1995; Helms 1999). Similarly, several studies have failed to detect the expected association between relatedness asymmetry and colony sex ratio (Pamilo and Seppä 1994; Vargo 1996; Brown and Keller 2000; Foitzik and Heinze 2000; Fournier et al. 2003). Three hypotheses have been proposed to account for queen manipulation of colony reproduction in ants. First, queens could prevent workers from discriminating against males by concealing the gender of their brood to gain control over reproductive decisions in the colony (Nonacs and Carlin 1990; Nonacs 1993). To our knowledge, however, this sexual deception hypothesis has not yet received experimental support. Second, queens may bias the primary sex ratio (the proportion of haploid-male eggs they lay) during the period in which sexuals are reared. Circumstantial evidence for such a queen effect comes from the monogynous form of the fire ant *Solenopsis invicta*, where it has been shown that queens can manipulate workers into raising male sexuals by limiting the number of female eggs laid (Passera et al. 2001). Third, queens might influence the sex ratio of reproductives by regulating the caste fate of the diploid eggs they produce. By primarily laying worker-destined eggs rather than queen-destined eggs, they could limit the number of female sexuals reared in a colony (Pamilo 1982). One potential mechanism queens could use to regulate the caste of their brood is sequestration of juvenile hormone (JH-III) in their eggs (Wheeler 1986). Such a maternal effect has been reported in the ant *Pheidole pallidula*, where topical application of JH-III to queens has been shown to promote sexualization of the female brood, while antiallatotropic substances (precocene II) prevent sexualization (Passera and Suzzoni 1978b; Passera and Suzzoni 1979; Passera 1982). Biometric analyses also showed that the volume and cell number of *corpora allata* of queens laying queen-biased eggs are significantly greater than in queens laying worker-biased eggs (Suzzoni 1983). However, there is still no empirical evidence that queens utilize this endocrine-mediated process to affect colony sex ratio.

We tested the hypothesis that queens influence the sex ratio in the ant *Pheidole pallidula*, and investigated two non-exclusive mechanisms that queens could use to regulate sex ratio. In this species, colonies may contain one or a few unrelated queens (Fournier et al. 2002) and workers that are completely sterile, so that relatedness asymmetry is uniformly maximal in single-queen (monogynous) and multiple-queen (polygynous) colonies (Boomsma 1993). Workers should, therefore, favor a 3:1 female-biased sex ratio in both colony types. Yet, in this species colonies produce primarily a single gender of reproductives (Keller et al. 1996). Sex specialization is tightly associated with the breeding structure, with monogynous colonies producing a male-biased brood and polygynous colonies almost exclusively a female-biased brood (Aron et al. 1999; Fournier et al. 2003). Here, we demonstrate that queens from monogynous colonies produce a significantly more male-biased primary sex ratio than queens from polygynous colonies. We also provide evidence that queens from monogynous colonies have lower rates of JH production and have a lower proportion of diploid eggs that develop into female sexuals than queens from polygynous colonies. These results are consistent with the "tragedy of the commons" hypothesis (Hardin 1968), which was proposed to account for sex ratio patterns in *P. pallidula* and other social insects (Wenseleers 2001; Fournier et al. 2003; Wenseleers and Ratnieks 2004).

Methods

In *P. pallidula*, there is no over-wintering brood (Bontpart 1964). Sexuals develop from eggs laid during the first days after queens resume egg laying in early spring, whereas all eggs laid later in the season develop into workers (Passera 1980). In this species, queens lay diploid eggs that are predestined to develop into workers or queens. This queen-worker caste determination occurs during oogenesis and appears to depend on JH-III produced by the queens (Passera and Suzzoni 1979; Passera 1980; Passera and Suzzoni 1984). Colony fragments of *P. pallidula*, including queens and workers (minors and soldiers) were collected in Bruniquel (Tarn-et-Garonne, France) in March 2003, before queens resumed egg laying. A sample of workers from each colony was stored in 95% ethanol for subsequent genetic analyses. Immediately after collection, queens from 27 randomly chosen colonies were used for measurement of individual rates of juvenile hormone production. An additional 69 colonies were transferred to the laboratory for sex ratio analyses.

Colony size and caste ratio of laboratory-reared colonies were standardized to one queen with approximately 525 workers (5% were soldiers; Passera 1977). They were kept at 27°C on a 12 h:12 h photoperiod, and were fed honey and mealworms twice a week. Eight days after they resumed ovipositing, queens were removed from the nests. This procedure enabled us to sample the very first eggs laid after overwintering, and avoided the effects of queen inhibition on sexual rearing by workers (Passera 1980). The number of eggs laid was counted, and about 70 eggs from each queen were stored at −80°C for primary sex

ratio determination. The remaining eggs were incubated with workers until they reached the second larval instar, at which point sexual and worker larvae are morphologically distinguishable (spherical versus vermiform, respectively; Passera and Suzzoni 1978a). The number of worker and sexual larvae were counted, and sexual larvae were stored at −80°C for sex ratio determination.

Thirty-two of the 69 colonies reared for sex ratio analyses produced sexual brood. From these, 18 colonies were randomly selected to test for a possible association between the primary sex ratio (numerical proportion of haploid eggs), the larval sex ratio (numerical proportion of larvae being haploid) and the breeding structure (monogyny or polygyny) of the colonies. The ploidy (haploid or diploid) of eggs and sexual larvae was determined from the DNA-content of nuclei from each sample by flow cytometry (Ploidy Analyser PAI, Partec), after DNA of individual nuclei was stained with DAPI fluorochrome (4′, 6-diamidino-2-phenylindole) (see Aron et al. 2003 for details). All sex determinations of eggs and larvae were performed blindly with regard to treatment, i.e. the person who analyzed ploidy levels did not know which colony was monogynous or polygynous.

The individual in vitro rate of juvenile hormone release, a good indicator of both hormone production rate and hemolymph titer in most insects, was determined for 27 queens using a rapid partition radiochemical assay (RCA) (Pratt and Tobe 1974; Tobe and Pratt 1974; modified by Feyereisen and Tobe 1981; Brent and Vargo 2003). The paired glands of the *corpora allata* and *corpora cardiaca* complex, hereafter referred to as the CA, were dissected under sterile conditions and cleaned of any attached tissue. Once excised, the CA was pre-incubated for 30 minutes in a Petri-dish at 26°C in 100 µl of modified TC199 medium (Specialty Media, Phillipsburg, NJ), with 50 mM Hepes buffer, pH 7.4, without methionine or bicarbonate, and containing 2% Ficoll 400 (Sigma Chemical Co.). Then, each CA was transferred to a 6×50 mm borosilicate culture tube containing 100 µl fresh medium supplemented with 5 µCi L-[methyl-3H]-methionine (specific activity of 70–85 Ci/mmol; NEN Life Science Products, Inc.). Glands were floated in the surface of the medium to ensure adequate oxygenation (Holbrook et al. 1997). Culture tubes were incubated at 26°C and were rotated at 90 rpm on a 15° pitch using an orbital shaker. Following incubation, radiolabeled JH was extracted from both the medium and CA with 250 µl ice-cold iso-octane. A 100 µl aliquot from each sample was evaporated under N_2, and mixed with 3 ml Scintiverse BD (Fisher) scintillation fluid. Radiolabeled methionine incorporation was measured using a scintillation counter (Beckman LS-5801). JH was verified as the principal product synthesized by the CA of *P. pallidula* by comparison to the synthetic products of *Blattella germanica* females as separated by thin-layer chromatography. *B. germanica* is known to produce primarily JH-III (Tobe et al. 1985). Pooled samples were analyzed on Whatman linear-K high performance silica gel plates (200 µ) using benzene, ethyl ether and acetic acid (84:15:1) as developing solvents. The proportion of JH-III from the total radioactivity was calculated as the ratio of the radioactivity in the JH zone in thin-layer chromatography and the radioactivity in an equivalent aliquot from the same extract after subtraction of blanks of ^{3}H-methionine. JH-III accounted for 44.8% of the total activity sampled. The remaining portion was primarily background radiation, but more polar compounds were also detected.

An appropriate incubation time for the RCA was established by measuring the time course of JH release over a 6-h period. During this period, hourly measurements were taken of the JH released by five individual incubated CAs. Each CA was transferred to new medium for each hour sampled. The cumulative rate of production was found to be highly linear over 6 h (r^2=0.79, p<0.05). A standard incubation time of 5 h was chosen to maximize the concentration of JH in the samples while ensuring an accurate assessment of the biosynthetic rates. Analyses of individual juvenile hormone biosynthesis rate were realized blindly with regard to treatment (i.e. breeding structure and sex ratios).

Because queens are extremely difficult to collect, the minimum queen number in each colony was inferred from the observed genotypes of workers at different loci. Three to five microsatellite loci (*Ppal-03*, *Ppal-12*, *Ppal-69*, *Ppal-83* and *Ppal-19T*; Fournier et al. 2002) were used to determine genotypes (mean number of workers analyzed per colony ± SD. = 10.2±1.4, n=57 colonies). In the study population, the number of alleles at these loci ranges from 10 to 16 and the expected heterozygosity ranges from 0.76 to 0.84 (Fournier et al. 2002). Individual ant DNA was extracted following a standard phenol/chloroform protocol (Sambrook and Russell 2001). Polymerase chain reactions (PCR) were carried out in a 10-µl volume as described in Fournier et al. (2002). Fluorescent PCR fragments were visualized by capillary electrophoresis on an ABI 3100 Genetic Analyzer (Applied Biosystem). Colony breeding structure could be determined without ambiguity since queens mate only once, and, in polygynous nests, queens are relatively few and almost invariably unrelated (Fournier et al. 2003).

To explore the effect of the breeding structure of the colonies (monogyny versus polygyny) on the primary and larval sex ratios, a logistic binomial regression model was carried out using the procedure GENMOD in SAS (version 8.2) (Boomsma and Nachman 2002). This model assumes that the dependent variable is a probability that follows a binomial distribution, and can account for variation in clutch size. The sex ratio was entered in the model as a dependent variable, and colony type (monogynous or polygynous) was entered as a qualitative variable. The same procedure was used to compare variation between the primary and larval sex ratios in monogynous and polygynous colonies, with the sex ratio as a dependent variable and development stage as a qualitative variable. The model scale parameter was adjusted to respond to over-dispersion ($s=\sqrt{D/\nu}>1$, where D is the deviance and ν is the degrees of freedom). All

mean values are given ± SE. All statistical tests were two-tailed.

Results

From the 18 colonies selected, genetic analyses revealed that six were monogynous and 12 were polygynous. Although the mean number of eggs laid per queen in monogynous colonies was lower than in polygynous colonies, the difference was not significant (743±103, range: 377–1043, n=6 and 1016±90, range: 563–1448, n=12, respectively; Mann-Whitney U-test, p=0.125; note that the small sample size results in a low statistical power of this test). However, the primary sex ratio produced was significantly different between queens from monogynous and polygynous colonies (48% and 6% haploid eggs, respectively; logistic binomial regression, Chi-Square = 10.31, df=1, p=0.001; Fig. 1).

The proportion of male larvae was almost eight times greater in monogynous colonies than in polygynous colonies (0.55±0.15, n=6 and 0.07±0.06, n=12, respectively; logistic binomial regression, Chi-square = 6.68, df=1, p<0.01; Fig. 1). Similarly, the average proportion of males among sexual larvae was higher in monogynous nests than in polygynous ones (0.82±0.07 and 0.09±0.06, respectively; logistic binomial regression, Chi-Square = 16.53, df=1, p<0.0001). The male-biased sex ratio in monogynous colonies could not be due to sperm depletion in some queens, as all queens also produced diploid brood. The strong bias of colony sex ratios among larvae was consistent with adult stage sex ratios previously found in the same study population (Fig. 1; Fournier et al. 2003), for both monogynous and polygynous colonies (proportion of males among sexuals at the larval stage versus at the adult stage: logistic binomial regression, Chi-square = 0.12, df=1, p>0.7 and Chi-square = 2.20, df=1, p>0.13, respectively).

No difference occurred between the proportion of haploid eggs laid by the queens and the proportion of male larvae reared among all castes, in both monogynous and polygynous colonies (Wilcoxon signed-rank paired test: monogynous colonies, W=−3, n=6, p=0.84; polygynous colonies: W=−5, n=12, p=0.69; Fig. 1). This indicates that workers rear eggs into larvae randomly with respect to the ploidy of the brood. In contrast, the mean proportion of diploid eggs developing into sexual rather than worker larvae was significantly higher in polygynous than in monogynous colonies (mean ± SE. = 0.78±0.10, n=12 and 0.30±0.18, n=5, respectively; logistic binomial regression, Chi-Square = 5.86, df=1, p=0.016; Fig. 2).

From the 27 queens for which the rate of juvenile hormone release from the CA was determined, 10 originated from monogynous colonies and 17 from polygynous ones. Mean release rates were about three times higher in queens from polygynous colonies than in those from monogynous colonies (1.279±0.311 pmol/h and 0.437±0.194 pmol/h, respectively; Mann-Whitney U-test, z=−2.109, p=0.035; Fig. 3).

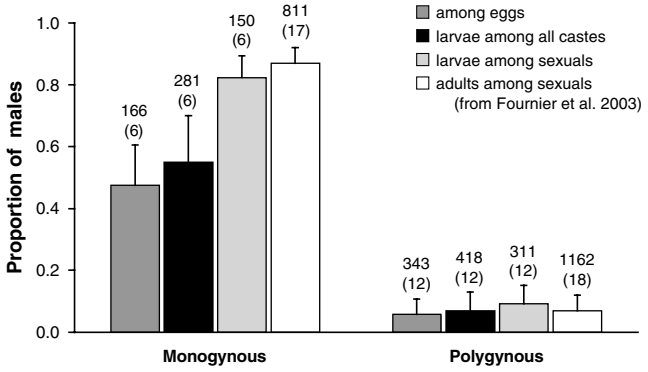

Fig. 1 Mean proportion (± SE.) of male (haploid) eggs laid by queens and of males reared among all larvae, among sexuals at the larval stage and among sexuals at the adult stage in monogynous and polygynous *P. pallidula* colonies. The number of individuals sampled and the number of colonies from which they were collected (within brackets) are reported above the error bars

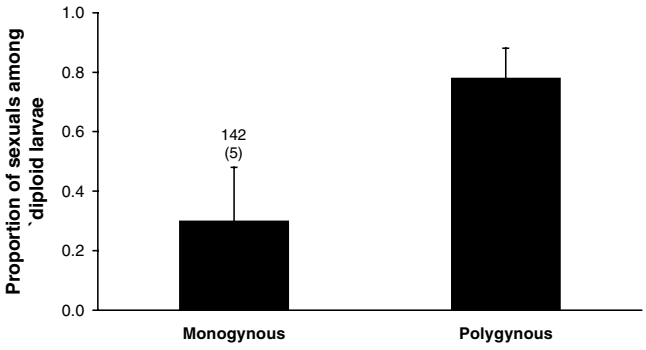

Fig. 2 Mean proportion (± SE.) of diploid eggs developing into sexual larvae in monogynous and polygynous colonies of *P. pallidula*. The number of larvae sampled and the number of colonies from which they were collected (within brackets) are reported above the error bars

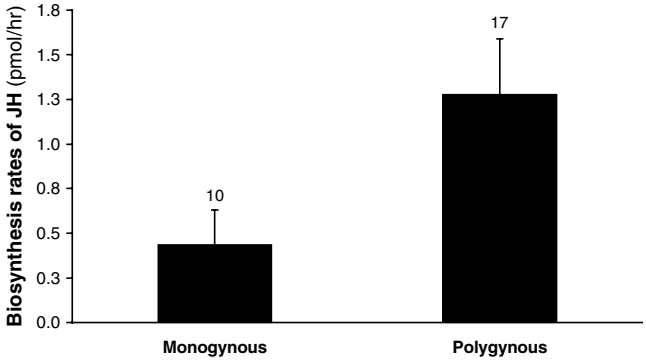

Fig. 3 Average biosynthetic rate of JH production (pmol/h) by queens from monogynous and polygynous *P. pallidula* colonies. A single queen was analyzed for each polygynous colony. Numbers of queens analyzed are reported above the error bars

Discussion

Our results show that queens of *P. pallidula* regulate colony sex ratios in two complementary ways: by determining the

proportion of female eggs laid and by hormonally biasing the development of female eggs into either a worker or reproductive form. We found that the primary sex ratio of queens from monogynous colonies is significantly more male-biased than that of queens from polygynous colonies, and that this ratio is sustained as eggs mature into second instar larvae. The possibility that our estimates of the primary sex ratio could be biased, e.g., by workers preferentially eliminating haploid eggs in polygynous colonies, seems unlikely. No study has demonstrated so far that queen-laid male eggs are treated differently from queen-laid female eggs in any ant species. Rather, it has been shown that workers perform sex-allocation biasing by killing male larvae but not eggs (Passera and Aron 1996; Chapuisat et al. 1997; Chapuisat and Keller 1999; Aron et al. 2004).

We also found that most diploid brood develop into sterile workers in monogynous colonies, whereas a large proportion of diploid brood develop into reproductive females in polygynous colonies. Queens from polygynous nests also produce JH at a significantly higher rate. The strong association between breeding structure, queen hormone level and the caste ratio of female brood supports the view that the reduced production of female sexuals in monogynous colonies stems from queens laying eggs hormonally promoted to develop into the worker caste. In contrast to other species of social Hymenoptera studied so far, where caste determination usually results from differential feeding at the larval stage (Wheeler 1986), caste determination in *P. pallidula* occurs during oogenesis and depends on JH-III produced by the queens (Passera and Suzzoni 1979; Passera 1980; Passera and Suzzoni 1984).

Our results for *P. pallidula* are, to our knowledge, the first to show that ant queens can use two complimentary mechanisms to exert substantial proximate control over the colony's secondary sex ratio. In this species, sexuals are reared from cohorts of eggs that are largely separated in time from eggs yielding workers (Bontpart 1964; Passera 1980), but the limited production of worker-destined eggs in early spring does not affect investment in colony maintenance (Reuter and Keller 2001; Roisin and Aron 2003). This separation in the periods of worker and sexual production gives queens great power to control sex ratio, and limits the power of workers to control sex allocation. Whether queens of other social insects can also control the caste of their brood at the egg stage, and the possible evolutionary significance of such a control mechanism, remain to be determined.

The first experimental evidence for queen control over sex allocation in ants recently came from the monogynous form of the fire ant *Solenopsis invicta*, in which the proportion of haploid eggs laid by the queen determines whether the colony will produce primarily male or female sexuals (Passera et al. 2001). In this species, queens of male-specialist colonies can dictate colony sex ratios by limiting the proportion of diploid-female eggs they produce. Similarly, in the ant *Pheidole desertorum*, specialization in male production might result from queens controlling the primary sex ratio, since workers preferentially rear female sexuals rather than males when given the opportunity (Helms et al. 2000). Helms (1999) hypothesized that queens of this species could also affect colony sex ratio by laying mostly worker-destined eggs in male-specialist colonies during the reproductive period, but he did not provide experimental support for this hypothesis. The ability of *P. pallidula* queens to vary primary sex ratio in response to the breeding structure, together with their hormonally-mediated influence over the caste development of female eggs, gives queens great power to determine the colony sex ratio.

Despite the ability of queens to exert some proximate control over brood sex allocation, evidence suggests that workers of *P. pallidula* may still have some measure of influence over sex investment ratios at the population level. First, sex investment ratios in populations lie between the equilibria values for queens and workers (1.1:1 to 1.8:1 in favor of females; Keller et al. 1996; Aron et al. 1999; Fournier et al. 2003), indicating that, at the population level, queens do not have full control over reproductive allocation. Second, in nature more than 70% of the male-specialist colonies produce a small number of reproductive females, while about 72% of female-specialist colonies never produce males (Keller et al. 1996). Primary sex ratio analyses showed, however, that queens from both colony types laid male eggs (see also this study), even though many colonies produce only female reproductive broods. Hence, queens of *P. pallidula* produce haploid eggs but workers do not rear males into adulthood in about one-third of the colonies, indicating that workers are able to recognize and selectively eliminate males at some point during brood development (Keller et al. 1996). Such a worker influence over the sex ratio was also suggested in *Pheidole desertorum*, where no males are found in female-specialist colonies but reproductive females are produced in male-specialist colonies (Helms 1999; Helms et al. 2000). Although the timing of male brood elimination still remains unknown in *P. pallidula*, the fact that the proportion of haploid males is sustained from egg to second instar larva strongly suggests that workers do not eliminate males before this developmental stage. This contrasts with previous studies on other ant species, showing that workers are able to discriminate between male and female brood at the first larval instar (*Linepithema humile*, Passera and Aron 1996; *Lasius niger*, Jemielity and Keller 2003; *Plagiolepis pygmaea*, Aron et al. 2004; but see Chapuisat et al. 1997 for late male elimination in *Formica exsecta*). Clearly, more experiments are needed to determine when workers can identify the sex of the brood and therefore bias sex ratio.

The strong associations between breeding structure, rate of JH production in individual queens and colony sex ratio provide new insights into the proximate mechanisms for the "tragedy of the commons" hypothesis (Fournier et al. 2003; Helms et al. 2004), which was proposed to account for the occurrence of split sex ratio in *P. pallidula*. Under this hypothesis, an evolutionary arms race in egg production would occur among queens in polygynous colonies, each queen preferring to be the mother of as many of the sexual offspring as possible. This competition would result in

increased individual egg production relative to queens from monogynous colonies. Our data show, however, that queens from the two colony types do not differ significantly in this respect. In *P. pallidula*, the reproductive success of competing queens may be more appropriately measured by the proportion of queen-destined eggs laid, rather than by queen fertility *sensu stricto*. Every queen in these colonies would therefore benefit from producing more queen-destined eggs than her reproductive competitors, a result which we have shown queens can achieve through hormonal manipulation. As a consequence, split sex ratio might result from a caste fate conflict among queens (Bourke and Ratnieks 1999; Wenseleers and Ratnieks 2004) in the context of a tragedy of the commons (Hardin 1968). The female bias in polygynous colonies would then also select for a compensating male bias in the monogyne colonies. In accordance with this hypothesis, previous experiments with this species showed a significant reproductive skew for the production of female sexuals among queens in polygyne colonies (Fournier et al. 2004).

In conclusion, although workers of *P. pallidula* have the ability to eliminate males and bias sex investment ratio, the magnitude of their control is largely constrained by the primary sex ratio together with the proportion of queen-destined eggs produced by queens. Evidence that sex ratio frequently is not associated with relatedness asymmetry and/or is less female-biased than the worker optimum in ants (Bourke and Franks 1995; Crozier and Pamilo 1996; Chapuisat and Keller 1999) suggests that such mechanisms allowing queens to influence sex ratio might be more common than previously thought. More generally, this study illustrates how the outcome of queen-worker conflicts over reproduction in social insects is determined by the party with the greatest power to act (Beekman and Ratnieks 2003).

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