

Insect. Soc. 51 (2004) 395–399
0020-1812/04/040395-05
DOI 10.1007/s00040-004-0758-9
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Research article

Evolution of miniaturisation in inquiline parasitic ants: Timing of male elimination in *Plagiolepis pygmaea*, the host of *Plagiolepis xene*

S. Aron¹, L. Passera² and L. Keller³

¹ Service d'Eco-Ethologie Evolutive, CP 160/12, Université Libre de Bruxelles, 50 av. F.D. Roosevelt, 1050 Brussels, Belgium, e-mail: saron@ulb.ac.be

² Centre de Recherches sur la Cognition Animale, Université Toulouse III, France, e-mail: passera@cict.fr

³ Institut d'Ecologie, Université de Lausanne, Switzerland, e-mail: Laurent.Keller@je-zea.unil.ch

Received 9 February 2004; revised 20 April 2004; accepted 22 April 2004.

Summary. Inquiline ant species are workerless social parasites whose queens rely completely on the host worker force to raise their brood. A remarkable feature of inquiline ants is the small size of sexuals, which are of the same size as or smaller than host workers. It has been suggested that miniaturisation evolved by parasites to prevent host workers from discriminating between their own worker brood and the inquiline sexuals, so that male and female inquilines can develop under conditions where the host species does not produce its own sexuals. In line with the miniaturisation hypothesis, workers of the ant *P. pygmaea* cull all the male brood of their own species, whereas at the same time males of their inquiline parasite *P. xene* are reared to adulthood. Here, we tested whether *P. pygmaea* workers recognize and eliminate males of their own species when they reach the size of the larger workers. Contrary to the assumption that size is indeed the primary cue used by workers to discriminate male from worker brood, we found that males of *P. pygmaea* are culled between the small and medium larval stages, that is much before reaching the critical size of the largest worker larvae. Based on this finding, we propose an extension of the miniaturisation hypothesis with a first step whereby the parasitic *P. xene* males escape the caste and sex recognition system of the host during early development. The most likely mechanism is chemical mimicry of host worker larvae. Miniaturisation would have evolved later to prevent the host workers to secondarily use size as a recognition cue to eliminate *P. xene* males.

Key words: Social parasitism, size reduction, brood sex ratio, ants, sexual deception.

Introduction

Social parasitism, whereby individuals of a species exploit parental care from another species, has evolved repeatedly in eusocial Hymenoptera and has been reported in over 250 species of ants, bees and wasps (Wilson, 1971; Wcislo, 1987; Hölldobler and Wilson, 1990). In its most extreme form, permanent inquilinism, social parasitism has been associated with the loss of the worker caste. The parasites thus completely rely on the worker force of the host to raise their brood. Inquilines queens infiltrate host colonies where they usually coexist with the host queen, and produce their sexual offspring that are reared by the host workers simultaneously with their own larvae.

One of the most distinctive traits associated with inquilinism in ants is the small size of male and female sexuals. It has been suggested that reduced body size evolved as a means to take advantage of the host caste determination system, so that parasite larvae can develop even when the host produces no sexuals (Bourke and Franks, 1991; Nonacs and Tobin, 1992). Specifically, the miniaturization hypothesis assumes that size is the critical cue used by host workers to discriminate between worker and sexual brood. Thus, the size of male and female inquilines would have decreased to that of the host workers so as to prevent workers of the host species to discriminate between their own worker brood and the inquiline sexuals. Findings of a recent study in the ant *Plagiolepis xene*, an inquiline parasitic species of the ant *Plagiolepis pygmaea*, were in line with predictions of the miniaturisation hypothesis (Aron et al., 1999). First, male and female *P. xene* pupae were significantly smaller than sexual pupae of their host but remarkably similar to the host worker pupae. Second, the coefficients of variation of male and female *P. xene* pupal length were nearly four times smaller than the coefficients for *P. pygmaea* workers, male and female sexuals, supporting the view that there is strong stabilizing selection on the size of the parasite brood. Third, we found that in early spring minia-

turisation of the parasite was indeed accompanied by its ability to develop into sexuals, even though the host actively prevented production of its own sexuals. The proportion of male eggs laid was very similar in the parasite *P. xene* and its host *P. pygmaea*. However, the *P. pygmaea* workers effectively eliminated all *P. pygmaea* males, while *P. xene* males were reared. Likewise, all *P. pygmaea* female larvae gave rise to workers, whereas *P. xene* females all developed into sexuals.

The aim of this study was to test one important, yet untested, assumption of the miniaturisation hypothesis, namely that host workers can discriminate between sexual and worker brood only once the sexuals become larger than the larger host workers. We therefore determined at which stage of their development *P. pygmaea* sexuals are recognized and eliminated by workers in early spring, at which time only *P. pygmaea* worker brood develops (Passera, 1969). Especially, we analysed brood sex ratio variation (proportion of males) at several developmental stages. If size is indeed the primary cue used by *P. pygmaea* workers to discriminate between workers and sexuals, one would predict that the *P. pygmaea* males which are permanently produced but only reared during the time of sexual production will be eliminated after they have reached the size of the largest worker larvae. Conversely, recognition and elimination of the male brood below this critical size threshold would not support the miniaturization hypothesis and demonstrate that host workers use other cues than size to identify males.

Materials and methods

Field collection and sampling

Twelve polygynous colonies of *P. pygmaea* were collected on March 11 and 14, 2000 (before queens resumed egg-laying) from the Lauragais region (Fendeilles, Aude, Southern France). At this period of the year, all the queens found in a nest are mated (Mercier et al., 1985). All the existing brood in the colonies was discarded. The colonies were maintained under standard laboratory conditions ($26 \pm 1^\circ\text{C}$) and fed on honey. Under these conditions, queens start to lay eggs and about 14% of these eggs were previously shown to be male (Aron et al., 1999; see Fig. 1). In this species, however, the eggs laid in spring never develop into sexuals (Passera, 1969), as both male and female sexuals develop in early July from overwintering brood only.

To determine the size at which males are eliminated, we analysed brood sex ratio (the proportion of males) at several development stages. To avoid any confounding effect due to temporal variation in the primary sex ratio among queen-laid eggs (e.g., see Aron et al., 1994), we studied the proportion of males in the same brood cohort. In *P. pygmaea*, worker larvae reach a mean size (\pm SD) of 1.38 ± 0.10 mm before pupation. Male and female larvae become larger, reaching respectively 1.59 ± 0.10 mm and 2.80 ± 0.13 mm before pupation (Aron et al., 1999). The mean size of fully-grown sexual larvae of the inquiline *P. xene* is 1.33 ± 0.02 mm for males and 1.35 ± 0.02 for female sexuals. Five worker larval instars can be recognized on the basis of their length (Passera, 1968). We determined the proportion of males for three larval classes: (i) small hairless larvae (<0.55 mm) which correspond to the first instar (mean number of larvae analysed per nest: $X \pm \text{SD} = 16.5 \pm 2.11$); (ii) medium larvae (size range: 0.90–1.15 mm), which include the third and fourth instars ($X \pm \text{SD} = 12.8 \pm 1.47$); and (iii) large larvae (1.15–1.35 mm), which is the size of the fifth worker larval instar ($X \pm \text{SD} = 11.3 \pm 1.42$). The latter size corresponds to the largest sexual pupae and larvae of the inquiline parasite *P. xene* (Aron et al., 1999). We also report the proportion of *P. pygmaea* males at the pupal stage.

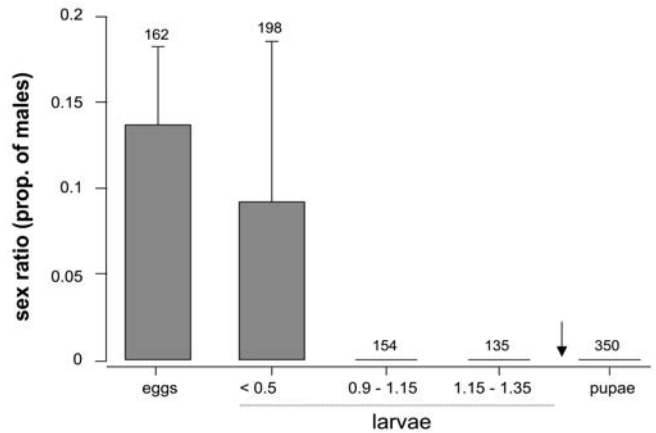


Figure 1. Sex ratio expressed as the numerical proportion of males (mean \pm sd) as a function of mean larval size in *Plagiolepis pygmaea*. Data on the sex ratio among eggs are taken from Aron et al. (1999). Larvae were sorted into three size classes: small (<0.55 mm), medium (0.90–1.15 mm) and large (1.15–1.35 mm). Sample sizes for which the gender was determined are reported above error bars ($n = 6$ colonies for eggs and $n = 12$ colonies for larvae and pupae). The arrow indicates the size at which males should be eliminated according to the miniaturisation hypothesis.

Brood sex ratio variation

In Hymenoptera, females are derived from fertilized diploid eggs, whereas males generally arise from unfertilized haploid eggs. In ants, the sex of larvae cannot be determined on the basis of external morphological characters. We therefore used flow cytometry to estimate the proportion of males in each of the three larval classes. Flow cytometry allows us to distinguish between males and females on the basis of their nuclei DNA content from the very first larval stage (i.e., immediately after egg hatching) (Aron et al., 2003). Larvae were chopped with a sharp razor blade in a petri dish to free interphase nuclei from the tissues. Nuclei were then stained according to Vindelov et al. (1983). Each individual was suspended successively in three solutions: (1) Trypsin (1.5 mg/50 ml buffer solution: 200 mg Trisodium citrate, $2\text{H}_2\text{O}$, 104.4 mg Sperminetetrahydrochloride, 12.1 mg Tris(hydroxymethyl)-aminomethane, 200 μl Igepal suspension, 200 ml dH_2O ; pH adjusted to 7.6), (2) Trypsin inhibitor + Ribonuclease A (25 mg + 5 mg/50 ml buffer solution) and (3) Propidium Iodide + Sperminetetrahydrochloride (20.8 mg + 58 mg/50 ml buffer solution). Ready solutions were kept in darkness at $+4^\circ\text{C}$ for 60 minutes before flow cytometric analysis.

Propidium Iodide (PI) stained nuclei from each larva were analysed separately on a FACSCALIBUR flow cytometer (Becton Dickinson, Erembodegem, Belgium). For each larva, the DNA content of >500 nuclei was analysed after exclusion of non-nuclear fluorescent debris and nuclear aggregates by pulse shape analysis (i.e., evaluation of fluorescence area vs. fluorescence width of the incoming signals; Aron et al., 2003). In some cases, two (or more) nuclei at the G0/G1 stages of the cell cycle stuck together. These aggregates could be identified (and discarded) because they took longer to pass through the laser beam resulting in a larger pulse width. Fluorescence-area frequency distributions of stained nuclei were retained and, since PI binds stoichiometrically to DNA, these distributions represent DNA ploidy distributions of the cells within every given sample.

Statistical analysis

Kruskal-Wallis nonparametric ANOVA tests were used for comparing the proportion of males in the different classes of brood (eggs and larvae). Multiple comparisons among pairs of means were performed by using Dunn's test (Zar, 1984).

Results

The flow cytometric analyses revealed that the proportion of males differed significantly across the larval developmental stages (Fig. 1; Kruskal-Wallis test, $H = 34.06$, $p < 0.0001$). It was significantly greater at the small larval stage than at the medium larval stage, large larval stage and the pupal stage (Dunn's multiple comparison test, all $p < 0.05$). In fact, not a single male was found in the medium and large larvae classes. Similarly, at the pupal stage no males were found, all pupae being workers. These data show that males were eliminated between the small and medium larval stage, that is much before male larvae reach the critical size of worker larvae before pupation.

The proportion of small male larvae varied significantly across the 12 colonies ($\chi^2_{11} = 21.70$, $p = 0.027$), males being present in six colonies only. The proportion of small male larvae in the colonies containing males was $X \pm SD = 0.15 \pm 0.06$ (range: 0.05–0.22). This value was not significantly different from the proportion of males at the egg stage (for the comparison with only the six colonies containing males at the small larval stage and the comparison with all 12 colonies; Kruskal-Wallis test, $H = 3.62$, $p = 0.16$).

Discussion

The results of this study show that a key assumption of the miniaturisation hypothesis, namely that size is the primary cue used by host workers to discriminate between sexual and worker brood, is not fulfilled in *P. pygmaea*. The comparison of sex ratio between the different developmental stages shows that workers cull males before they reach the medium size larval class (0.90–1.15 mm). This threshold is much lower than the size that worker larvae reach before pupation (1.38 ± 0.10 mm). Thus, workers clearly use other cues than the size difference between males and workers to identify and eliminate male brood.

These findings raise the question of why the transition from non-parasitic to parasitic habits has been accompanied by a drastic size reduction of *P. xene* sexuals. The first possibility is that size reduction occurred for other reasons than those proposed by the miniaturisation hypothesis. An important factor influencing the size of ant queens is the mode of colony founding. In some species, queens rely on their own body reserves (fat and storage proteins) to raise the first brood and start a new colony independently. By contrast, in some other species young queens are adopted into an established colony after mating and found a new colony dependently. These two strategies are associated with marked morphological differences between queens, with queens using independent colony founding being typically larger than those using dependent colony founding (Keller and Passera, 1989; Keller and Ross, 1993; McInnes and Tschinkel, 1995; Stille, 1996; Ruppell et al., 1998; Heinze and Keller, 2000). Thus, the evolution of inquilineism may have favoured a size reduction of female sexuals since it has also been accompanied by a shift from independent to dependent colony founding. In addition,

mating takes place within the natal nest where *P. xene* queens remain when fertilized (Passera, 1969). This reduced dispersal syndrome is frequently observed in advanced inquiline social parasites (Hölldobler and Wilson, 1990). However, several elements do not support the hypothesis that size reduction merely results from a change in the mode of colony founding. First, although queens using dependent colony founding are usually smaller than queens founding independently, they still remain significantly larger than the workers of their own species. By contrast, the size of *P. xene* queens has decreased to the level of *P. pygmaea* workers (Aron et al., 1999). Similarly, a comparative study showed that queens of workerless inquiline ants are of the same size or smaller than host workers in 18 out of 19 species (Nonacs and Tobin, 1992). Second, the evolution of dependent colony founding does not provide a good explanation for the very low variance in the size of *P. xene* sexuals (Aron et al., 1999). Finally, this hypothesis also fails to account for a reduction of male size since there is no direct correlation between male size and mode of colony founding in ants. In short, although size reduction may have been favoured by the evolution of dependent colony founding, this hypothesis fails to account for the extreme miniaturization of sexuals in *P. xene*.

A more plausible explanation for the miniaturisation of *P. xene* sexuals is that it evolved in a two-step process involving an arms race between the host and parasite. The first step would have been the ability of the parasite to circumvent the system of caste discrimination of the host. It is likely that *P. pygmaea* workers' ability to identify males of their own species early in development is based on odour differences between male and female larvae. Although chemical differences between male and female larvae have not been demonstrated so far in ants, several studies have shown that workers can discriminate between adults of different castes and sex on the basis of hydrocarbon profiles (Monnin et al., 1998; Liebig et al., 2000; Cuvillier-Hot et al., 2001; Heinze et al., 2002). Hence, the ability of *P. xene* males to escape recognition would be explained if they evolved the ability to effectively mimic the odour of *P. pygmaea* female brood, as has been shown recently in the non parasitic ant *Cardiocondyla obscurior* (Cremer et al., 2002; see also Sumner et al., 2003 for a thorough discussion on this issue). In that respect, it is interesting to note that social parasites have repeatedly been able to overcome the odour-based nestmate discrimination code of their hosts to achieve social integration in a host colonies (see review of Lenoir et al., 1999, 2001; D'Ettorre et al., 2002). However, once the parasite has been able to overcome the chemical system of caste recognition, host workers may have responded by using size as a cue, killing *P. xene* males when these became larger than the *P. pygmaea* workers. This, in turn, would have led to selection on *P. xene* males to reduce size to below that of *P. pygmaea* workers to escape detection. This hypothesis of joint chemical and size mimicry is an extension of the miniaturisation hypothesis. The inclusion of the chemical mimicry component makes the hypothesis consistent with our findings that *P. pygmaea* workers recognize and eliminate *P. pygmaea* males, but not *P. xene* males. A comparison of the chemical profile of para-

site and host larvae of various castes would be of great interest to test this hypothesis. Unfortunately, such data are impossible to obtain to date, because it is not possible to discriminate the parasite from the host workers on the basis of external characters before the adult stage.

The findings of this study are also relevant with regard to the hypothesis of sexual deception, which states that queens may conceal the gender of their brood to gain control over reproductive decisions in the colony (Nonacs and Carlin, 1990; Nonacs, 1993). In colonies of social insects there are two types of queen-worker conflicts. The first relates to sex ratio with worker preferring a more female-biased sex ratio investment than queens which benefit from equal investment into males and females (Trivers and Hare, 1976). One of the proximate mechanisms used by workers to bias sex ratio toward more females is elimination of males (Aron et al., 1994; Sundström et al., 1996; Chapuisat et al., 1997). Thus, uncertainty about gender should prevent workers from culling males (and thus benefit queens) because mistakenly destroying related females could be very costly. The second type of queen-worker conflict relates to male parentage. In *P. pygmaea* and some other ant species, workers are not sterile and may increase their fitness by laying male eggs. Sexual deception might enable the queen to monopolize male parentage. Indeed, if workers can recognize males only at an advanced stage of development, the workers' fitness returns from raising their brothers to adulthood might be higher than that of replacing them with sons (Nonacs and Carlin, 1990). Our finding that *P. pygmaea* workers recognize and eliminate males of their own species early during development provides strong evidence against the sexual deception hypothesis.

Another interesting finding of this study is that the proportion of males at the small larval stage varied significantly across colonies. Half of the colonies contained no males, whereas the other half contained about 15% males. There are two possible explanations for this pattern. First, workers had already eliminated males in the six colonies where no males were found. Alternatively, queens may produce a variable primary sex ratio with about half the queens producing no males. Differences in the primary sex ratio laid by queens according to the social environment and/or ecological conditions have been reported at least in three other ant species, the Argentine ant *Linepithema humile* (Keller et al., 1996), the black ant *Lasius niger* (Aron and Passera, 1999) and the fire ant *Solenopsis invicta* (Passera et al., 2001).

In conclusion, this study demonstrates that *P. pygmaea* workers can recognize and eliminate unwanted male larvae of their own species when their size is much smaller than that reached by the largest worker larvae. This indicates that workers use cues other than size to determine the sex of the brood of their own species. Thus, the apparent ability of males of the inquiline social parasite *P. xene* to escape similar culling by the host workers of *P. pygmaea* cannot be explained by their size miniaturisation alone, but also requires the ability of *P. xene* males to remain undetected during the early larval stage, mostly likely by mimicking the odour of the host workers.

Acknowledgements

We thank R. Crozier for suggesting this experiment. Thanks to R. Crozier, M. Chapuisat, J. Parker, R. Hammond and two anonymous referees for their comments on the manuscript. Financial support was provided by the Belgian National Fund for Scientific Research (FNRS) through personal support (Senior Research Associate position) and several grants (to SA), the Université Paul Sabatier (to LP) and several grants from the Swiss National Science Foundation (to LK).

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