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Sex allocation decision under superparasitism by the parasitoid wasp *Eupelmus vuilleti*.

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Running head: Sex allocation decision in a parasitoid wasp.

Abstract:

Superparasitism is a widespread phenomenon in parasitoids and may be advantageous in some circumstances. In this study, offspring sex ratio was analysed in three superparasitism situations: when the second egg was laid by a random *Eupelmus vuilleti* (Hymenoptera: Eupelmidae) female from a group, when an isolated female was allowed to lay two eggs on the same host (self-superparasitism) or laid one egg on a host already parasitized by a conspecific (conspecific superparasitism). Females produced a different offspring sex ratio according to the different superparasitism situations tested. These sex ratios are in line with the Local Mate Competition theory. The results further suggest that females can discriminate between hosts parasitized by a conspecific or by themselves and adapt the sex of the eggs they lay accordingly.

Key words: self- and conspecific superparasitism, host discrimination, reproductive strategies, Hymenoptera, Eupelmidae.

Introduction

In solitary ectoparasitoids, two or more eggs laid on the same host lead to competition between the larvae and result in all but one dying. Any supernumerary eggs are eliminated by physical larval combats, physiological suppression, or scramble competition (Mayhew and Hardy, 1998). Solitary parasitoids tend thus to maximize their own fitness by ovipositing on the best quality hosts (i.e. unparasitized) for the survival of their offspring (Charnov and Stephens, 1988). Most parasitoid females can avoid superparasitism (i.e. laying an egg on a host already parasitized) if they can recognize that a host has previously been parasitized (host discrimination). Consequently, superparasitism was thought to result from oviposition mistakes, because females were not expected to waste time or offspring (van Lenteren, 1981). However, while the probability of survival of supernumerary eggs may be low, theoretical arguments propose that there may be conditions under which superparasitism is adaptive (van Alphen and Nell, 1982; Waage, 1986). Superparasitism can be advantageous if the number of unparasitized hosts is low in the environment or when the travel time between patches (reproductive area) is long (van Alphen and Visser, 1990). This of course assumes that the second egg has some chance of survival (van Baaren and Nénon, 1996).

The potential fitness gain when superparasitizing may depend on the maternal origin of the first egg present on the host. In conspecific superparasitism, an egg is laid on a host previously parasitized by a conspecific female, while in self-superparasitism it is laid on a host already parasitized by the same female (Waage, 1986). The fitness gain for an egg laid in self-superparasitism could be lower than in conspecific superparasitism (van Dijken and Waage, 1987) because a female which lays two eggs on the same host may obtain just one offspring after competition between its own progeny. However, self-superparasitism might still be advantageous when two or more eggs on a host increase the probability of gaining an offspring from that host, for example when the first egg laid dies during its development

because of non-viability or host defence reaction (van Alphen and Visser, 1990; Rosenheim and Hongkham, 1996). This pay-off could also depend on the probability of subsequent superparasitism by a conspecific female (Visser, 1993). Self-superparasitism may prevent conspecific superparasitism when a female is able to detect and count eggs already present on the host (van Alphen and Visser, 1990; Visser et al., 1990).

Females can adjust their offspring sex ratio to environmental conditions in order to maximise their reproductive success (Charnov, 1982; Godfray, 1994). The Local Mate Competition (LMC) model (Hamilton, 1967; Werren, 1980) provides an evolutionary explanation for the influence of superparasitism and parasitoid density on offspring sex ratio (Godfray, 1994). LMC predicts that when mating takes place on the emergence patch before the dispersion of daughters, gregarious parasitoid females exploiting that patch will deposit eggs with a female-biased sex ratio. Likewise, LMC predicts also that females will deposit eggs with a larger proportion of sons in previously parasitized hosts than in unparasitized hosts (Werren, 1980; Shuker et al., 2005). Predictions are similar if females visit a reproductive patch simultaneously in groups, or alone but sequentially (Godfray, 1994). Females may assess the number of other females present in the same ovipositing area by the frequency of contacts with them, or by the frequency of contacts with parasitized hosts or marks left by these other females (Godfray, 1994). In some species, females adjust the sex ratio deposited in both situations (*Nasonia vitripennis*, Wylie, 1976; King and Skinner, 1991; Godfray, 1994; Hardy, 2002; Shuker and West, 2004; Shuker et al., 2006), while in others they do not modify the sex ratio after contact with conspecifics (*Trichogramma evanescens*, Waage and Lane, 1984) or with parasitized hosts (*Telenomus heliothidis*, Strand, 1988).

In solitary parasitoids, LMC can influence offspring sex ratio when hosts are clumped (Godfray, 1994). In the case of superparasitism by solitary parasitoids, LMC could be less relevant than in gregarious parasitoids as only one egg can complete its development. In this

situation, mated females may favour a sex ratio bias towards the sex which is the better larval competitor (van Dijken and Waage, 1987; King, 1987). Different studies with different parasitoid species have found either a more male-biased sex ratio (*Gregopimpla himalayensis*, Shiga and Nakanishi, 1968) or a female-biased sex ratio (*Bracon hebetor*, Galloway and Grant, 1989; alysiine parasitoids, Mayhew and van Alphen, 1999; *Eupelmus vuilleti*, Darrouzet et al., 2003, 2007).

In *Eupelmus vuilleti* (CRW) (Hymenoptera, Eupelmidae), a solitary ectoparasitoid of the larvae and pupae of the Coleoptera Bruchidae *Callosobruchus maculatus* (F.), the offspring number is reduced and the sex ratio is more male-biased when self-superparasitism increases (Darrouzet et al., 2002). This sex ratio shift is mainly due to high female offspring mortality, because the number of two-female egg clutches was higher than two-male and mixed egg clutches suggesting that self-superparasitism could contribute to the variability of offspring sex ratios (Darrouzet et al., 2003). However, the response of females of this solitary ectoparasitoid to self-superparasitism raises several questions. Does the simultaneous presence of conspecific females in the same patch affect the offspring sex ratio of a particular female? Does an isolated female produce a different offspring sex ratio under self- and conspecific superparasitism? Is there any evidence that females are able to recognise hosts previously parasitized by themselves or by a conspecific female? Darrouzet et al. (2007) recently demonstrated that *E. vuilleti* females are able to discriminate parasitized hosts and unparasitized hosts.

The aim of the present study was thus to analyse offspring sex ratios in *E. vuilleti* under different superparasitism situations and investigate whether females adapt these ratios as predicted by theory. These superparasitism situations were investigated factoring in both the influence of the density of foraging females and the maternal origin of the first egg laid.

These two factors were chosen because of their potential influence on the female's sex allocation decision.

Materials and methods

Rearing conditions

The bruchid (*Callosobruchus maculatus*) and the parasitoid wasp (*Eupelmus vuilleti*) adults were collected from cowpea cultures from Burkina Faso at the end of the rainy season in October 1997. They were brought back to the laboratory and mass-reared in climatic chambers under conditions close to those of their origin zone: 12h 33°C:12h 23°C; L12:D12; 70% r.h. (Darrouzet et al., 2002).

Superparasitism experiments

The sex of each egg laid under superparasitism conditions was analysed using an experimental system in which gelatine capsules mimic seeds (Gauthier et al., 1996; Darrouzet et al., 2003, 2007). Such information is difficult to obtain when parasitoid development occurs within the seed. This artificial system mimics the bruchid pupal chamber in the seed and enables the egg-laying activity of *E. vuilleti* females (Darrouzet et al., 2003, 2007).

1) Superparasitism experiments with groups of ten females

a) Sex ratio deposited under superparasitism

To determine the sex of each of two eggs laid under superparasitism (i.e. the sex ratio decision made by a random female in a group at oviposition), a group of ten gelatine capsules each containing one *C. maculatus* L₄ larva was offered to a group of ten mated *E. vuilleti* females confined to an arena (diameter = 8 cm, height = 2.5 cm) containing the capsules. The bruchid L₄ larvae were placed inside the capsules after removal from seeds by dissection and

selection by size (9.12 ± 0.20 mg). Four replicated experiments of ten ovipositing females each were performed at the same time and ovipositions were observed in a rearing chamber (33°C : 23°C , 12L: 12D, r.h. 70%). Experiments were performed for one week during which females presented substantial egg-laying activity and did not run out of sperm in the spermatheca (Darrouzet et al., 2002). There were two four-hour exposure periods every day, and the parasitism behaviour of females was analysed at the end of each period. Every time two eggs were laid on the same host, each egg was transferred onto a different unparasitized host and deposited in a different cell in a Plexiglas sheet closed by a Plexiglas cover-slide until emergence of the parasitoid adults. Sex ratio calculations were carried out only when both eggs developed successfully. The sex ratio was measured as the proportion of females emerging from the cells in the Plexiglas sheets, i.e. the secondary sex ratio. At the end of each four-hour exposure period, entire patches were prepared with new capsules.

At the end of each experimental day, to determine the sex of all eggs laid in absence of superparasitism, we transferred each egg laid in parasitism onto its host in a Plexiglas sheet until emergence.

b) Sex ratio at emergence under superparasitism

In order to determine the sex of the individual winning the larval competition, eight groups of ten inseminated females were each offered ten gelatine capsules daily (two four-hour exposure periods), with the same experimental procedure previously described. In this experiment, each time two eggs were laid on the same host, the host and the two parasitoid eggs were deposited together in a cell in a Plexiglas sheet until emergence of the surviving parasitoid adult.

2) Superparasitism experiments with individual females

a) Sex ratio deposited under self-superparasitism

To determine the sex of each egg laid in self-superparasitism, sixteen mated females were offered individually and daily (two four-hour periods) five capsules each containing one host. Each time two eggs were laid on the same host, each egg was transferred onto a different host and deposited in a Plexiglas sheet as previously described.

At the end of each experimental day, we determined the sex of all eggs laid in parasitism by transferring them onto their host in a Plexiglas sheet until emergence.

b) Sex ratio deposited under conspecific superparasitism

To determine the sex of an egg laid in conspecific superparasitism, a patch of ten gelatine capsules each containing one *C. maculatus* L₄ larva was offered in the morning to ten virgin *E. vuilleti* females confined to an arena. Nine replicated experiments were performed. Capsules with parasitized hosts were isolated at midday. In the afternoon, five of these capsules, each containing one parasitized host, were offered to an isolated mated female (n = 15 replicates). When a mated female laid one egg, the two eggs on the same host were transferred onto a different host and deposited in a Plexiglas sheet to obtain the sex of the two parasitoid adults. Sex ratio calculations were carried out only when both eggs laid on the same host developed successfully.

Calculations and statistical analysis

To determine if the sex of each egg laid in superparasitism was randomized, we performed an expected binomial frequency. When laying a single egg on a host, *E. vuilleti* produces an unbiased sex ratio (see Results). If the sex ratio does not change and two eggs are laid per host, then we would expect half the hosts to have a mixed-sex brood (one fertilized, i.e. female, and one unfertilized, i.e. male egg), a quarter of the hosts to have two female eggs and

a quarter to have two male eggs. In superparasitism, the sex ratio of surviving adults was 0.51 (see Results). Thus, if sex were allocated to eggs at random, but with a mean proportion of 0.51 females, when two eggs are laid per host we would expect proportions of 0.26 hosts (0.51×0.51) to bear two females, 0.24 hosts (0.49×0.49) to bear two males and 0.50 hosts ($[0.49 \times 0.51] + [0.51 \times 0.49]$) to bear a mixed-sex brood. These proportions were multiplied by our sample size to give the expected numbers. This mean sex-ratio gave us an expected binomial frequency distribution of sex ratio. This binomial distribution was then compared with the experimental data (Figure 1). Experimental data were taken into account only when the two eggs laid together and then transferred onto a different host developed successfully.

χ^2 -tests were used to compare the binomial expectation and the experimental data for egg-sex combinations produced by groups of females, the egg-sex combinations between single and groups of females, and to analyse if offspring sex ratios were unbiased (Wilson and Hardy, 2002). A non-parametric design (Mann-Whitney U-test) was used to analyse the different offspring sex ratios. Analyses were carried out with Statistica software (Statsoft Inc.). A level of 5% was used throughout to determine significance.

Results

Sex allocation by groups of ten females.

E. vuilleti females in groups laid two eggs on the same host with an unbiased sex ratio $SR = 0.57 \pm 0.05$ ($n = 10$ groups of ten females which laid 204 pairs of eggs; $\chi^2 = 13.38$, d.f. = 9, $P = 0.14$). These females preferentially laid two eggs of the same sex on the same host (61.76% of clutches), and in 37.75% of cases two female eggs. These observed clutch percentages in a female population differed from those expected from a binomial distribution ($\chi^2 = 7.6$, d.f. = 2, $P < 0.025$) (Figure 1).

In 8.65% of clutches, both eggs laid together failed to develop, and in 32.56% just one egg failed to develop.

Sex of surviving progeny laid in superparasitism by groups of ten females.

After larval fights, the sex ratio of surviving parasitoids was unbiased ($SR = 0.51 \pm 0.07$; $n = 4$ patches of 10 females which laid 138 pairs of eggs; $\chi^2 = 2.57$, d.f. = 3, $P = 0.27$) and was not different from the SR of eggs before the larval fights (0.51 ± 0.07 vs. 0.57 ± 0.05 , Mann-Whitney U-test: $Z=0.51$, $P=0.69$).

In 11.54% of superparasitism cases, both eggs failed to develop.

Sex of eggs laid in parasitism by groups of ten females and individual females.

Females in groups parasitized host with an unbiased sex ratio ($SR = 0.46 \pm 0.03$; $n = 12$ patches of 10 females which parasitized 263 hosts; $\chi^2 = 9.78$, d.f. = 11, $P = 0.55$). This sex ratio is different from that produced by isolated females ($SR = 0.63 \pm 0.04$, $n = 15$ isolated females which parasitized 259 hosts; Mann-Whitney U-test: $Z=-2.31$, $P=0.02$) which is female biased ($\chi^2 = 25.69$, d.f. = 14, $P = 0.03$).

22.87% of eggs laid in parasitism failed to develop when ovipositing females were in groups, and 16.45% in the case of isolated females.

Sex of eggs laid in self- and conspecific superparasitism by individual females.

Isolated *E. vuilleti* females produced a female-biased sex ratio in self-superparasitism ($SR = 0.68 \pm 0.05$, $n = 16$ females which laid 154 pairs of eggs; $\chi^2 = 47.29$, d.f. = 15, $P < 0.005$). Females laid one egg with an unbiased sex ratio on a host already parasitized by a conspecific virgin female ($SR = 0.51 \pm 0.06$, $n = 15$ females which superparasitized 142 hosts; $\chi^2 = 15.04$,

d.f. = 14, $P = 0.37$). The two sex ratios obtained in these two superparasitism conditions are significantly different (Mann-Whitney U-test: $Z = -2.52$, $P = 0.01$).

In 6.24% of superparasitism cases, both eggs of the same pair failed to develop, and in 37.55% of cases, one egg failed to develop.

Comparison of sex combinations between individual females and random females from a group

The egg-sex combinations observed in self-superparasitism (eggs laid by isolated females) differed from those obtained in a group ($\chi^2 = 13.19$, d.f. = 2, $P < 0.005$) (Figure 2). The percentage of two-female-egg clutches was 1.65 times greater in self-superparasitism than in superparasitism with random females from a group.

Discussion

When in groups, *E. vuilleti* females produced an unbiased offspring sex ratio from parasitized and superparasitized hosts. Isolated females produced a female-biased sex ratio in parasitism and self-superparasitism, but an unbiased offspring sex ratio in conspecific superparasitism. These patterns of sex ratio behaviour in this species are consistent with the predictions of the LMC model (Hamilton, 1967; Werren, 1980; Shuker et al., 2005). In different parasitoid species, females increase the proportion of their sons when other females are present (King, 1987, 1993) with a predicted offspring sex ratio approaching equality ($SR = 0.5$). A conspecific egg on a host could also inform an isolated ovipositing female of the presence of putative conspecific females even if she never encounters them. In this situation, a female can alter her sex allocation as predicted by LMC (Shuker and West, 2004; Shuker et al., 2006). *E. vuilleti* females deposited a sex ratio in situation of conspecific superparasitism that differed from one predicted under a binomial distribution. The sex ratio deposited under

superparasitism by random females in a group was different from what was found for isolated females under self-superparasitism (see also Darrouzet et al., 2003). Overall, these results suggest that *E. vuilleti* females can control the egg-sex combinations in the different superparasitism situations analyzed here and in an earlier study.

In gregarious parasitoids, the sex ratio of eggs laid by the first female is usually female biased, while for eggs laid in conspecific superparasitism by a second female the sex ratio is male biased (Werren, 1980; Shuker et al., 2005). Therefore, males hatching from the eggs of the second female have a good chance of mating with females hatching from the first clutch. The second female can thereby increase the chance of propagating her genes at a low energy cost. For a solitary parasitoid, some fitness gain could be expected from the second egg when hosts are clumped (Godfray, 1994) and if the second egg laid in conspecific superparasitism has a high chance of developing as a result of the first egg dying during larval fights, by ovicide or during development due to egg non-viability.

Superparasitism has often been linked to a competitive superiority of one sex (King, 1987). This phenomenon tends to favour the sex which is the better larval competitor via a biased sex ratio (Suzuki et al., 1984; van Dijken and Waage, 1987). In *E. vuilleti*, we cannot determine morphologically which sex is the better competitor as we are unable to sex individuals in the early larval stages, i.e. at moments of larval fights, no non-invasive technique being available to sex each individual before larval fights. However, it is possible to sex individuals after larval fights by determining ploidy levels, either by counting the chromosomes (Ueno and Tanaka, 1997) or by flow cytometry (Aron et al., 2003). These ploidy techniques were not used in the present work and we simply analysed the sex of survivors at their adult stage. However, it is possible to calculate the putative sex ratios of the surviving adults according to which sex is the better competitor (Darrouzet et al., 2003). After analyzing results of egg-transfer experiments after oviposition by random females from a

group (figure 1), if female larvae are the better competitors, the sex ratio of surviving offspring will be 0.76; if males are the better competitors, the sex ratio will be 0.38; and if male and female have equal chances of winning the competition, the sex ratio will be 0.57. The sex ratio of surviving adults in superparasitism experiments without egg transfer was 0.51, suggesting that neither males nor females have competitive superiority. This result differs from those obtained by Darrouzet et al. (2003) in self-superparasitism conditions and from which the authors suggested that females were the better competitors. The difference observed in this species about which sex is the better competitor could be linked to the genetic origin of the competitors. When there are mixed-sex clutches in self-superparasitism, larval fights take place between siblings, whereas in superparasitism by a group of females, these fights probably occur between two unrelated larvae. In this case, it is possible that during conspecific fights between the two sexes, some larval females are not good competitors (because of a delay in hatching or a large difference in size for example) and female eggs are thus eliminated by larval males.

Some points about our experimental design need to be clarified. In our conspecific superparasitism experiment, each egg initially laid on hosts was male (they were produced by virgin females) and the sex ratio of eggs laid in conspecific superparasitism by isolated mated females is only relevant to this situation. If the first eggs were females, a different sex ratio could potentially be obtained. This supposes that females could discriminate the sex of an egg present on a host and adapt their reproductive strategies (laying or not laying an egg on the parasitized host, laying a male or a female). Further studies are required to verify this hypothesis. We were also unable to distinguish self- and conspecific superparasitism obtained in superparasitism by a group of females. We can assume that both these superparasitism conditions occurred and further studies are also required to analyse this point. Finally, the

time delay between the first and second egg laid may differ between a group versus a single female situation. This time delay could also influence the sex ratio decision of a female.

The major finding of the present study is that *E. vuilleti* females produce different offspring sex ratios according to the different parasitism and superparasitism situations analysed and that these sex ratio patterns are consistent with the LMC theory. This confirms our recent observation that females can discriminate between unparasitized and parasitized hosts (Darrouzet et al., 2007) and suggests that females can probably discriminate between hosts parasitized by a conspecific female or by themselves. Most parasitoid females are able to discriminate between unparasitized and parasitized hosts (an ability referred to as host discrimination) (Islam and Copland, 2000). The level of marker recognition could vary among species, some females being able to discriminate between hosts parasitized by their own eggs and those by conspecific eggs (van Dijken and Waage, 1987; Völkl and Mackauer, 1990; Ueno, 1994), while others seem unable to do so (Bai and Mackauer, 1990). However, neither the source nor the nature of the host-discrimination marker is known in *E. vuilleti* species and further studies are required.

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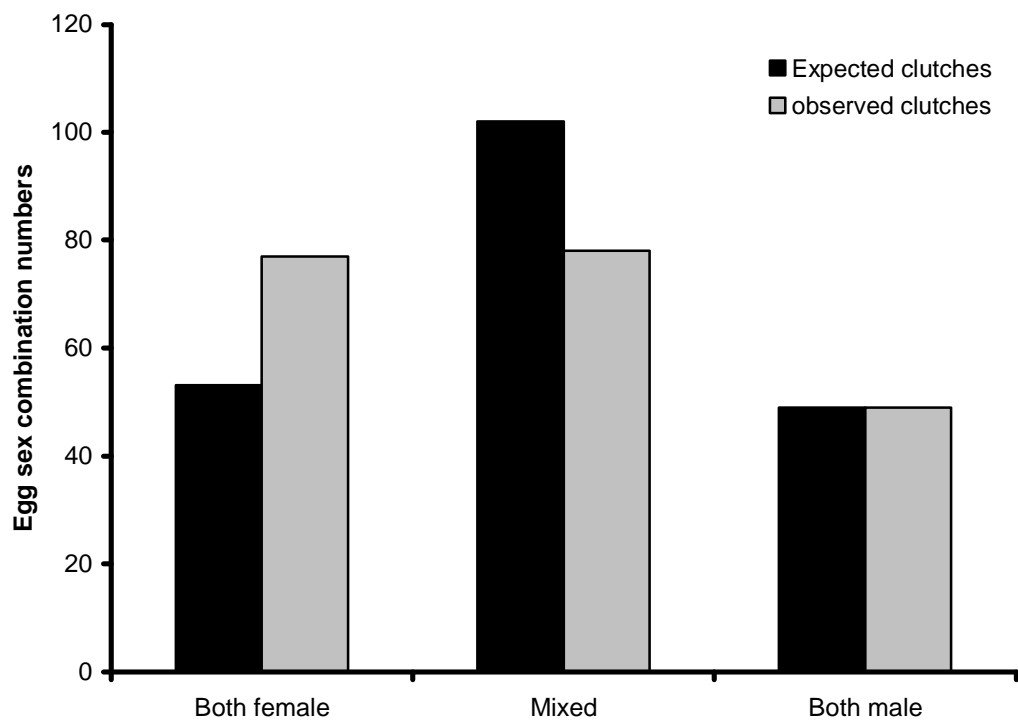
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Figure 1 Sex allocation decisions of four groups composed of ten mated *Eupelmus vuilleti* females laying two eggs per host in superparasitism (n = 204 pairs of eggs). Histograms in black indicate the expected clutches in a binomial sex ratio distribution = 0.51 (see Results), and histograms in grey the observed clutches.

Figure 2 Comparison of egg-sex combinations of two eggs laid in superparasitism by a group or by isolated *Eupelmus vuilleti* females. Histograms in black indicate the sex allocation decisions of 16 isolated mated females laying two eggs per host in self-superparasitism (n = 141 pairs of eggs) and histograms in grey the sex allocation decisions of four groups composed of ten mated females laying two eggs per host in superparasitism (n = 204 pairs of eggs).

416 Figure 1:

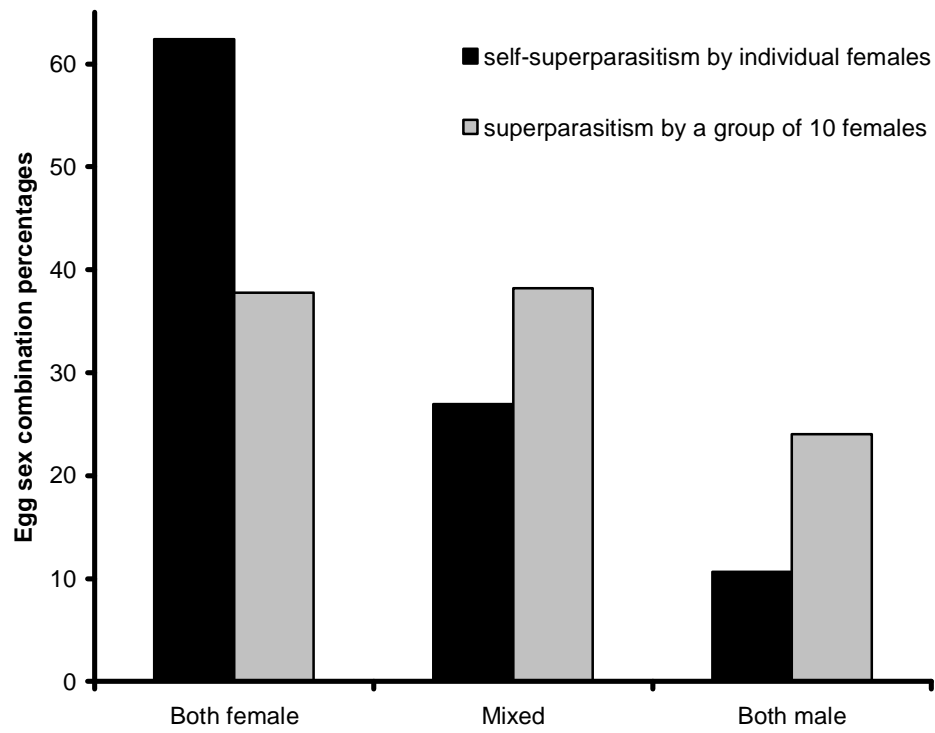
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419 Figure 2:



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