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

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8

9 **Running head:** Sex allocation decision in a parasitoid wasp.

10

11 **Abstract:**

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

22

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

## 25 **Introduction**

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

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

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

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

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

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

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

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

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

101

## 102 **Materials and methods**

### 103 *Rearing conditions*

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

109

### 110 *Superparasitism experiments*

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

116

#### 117 *1) Superparasitism experiments with groups of ten females*

##### 118 *a) Sex ratio deposited under superparasitism*

119 | To determine the sex of each of two eggs laid under superparasitism\_(i.e. the sex ratio  
120 | decision made by a random female in a group at oviposition), a group of ten gelatine capsules  
121 | each containing one *C. maculatus* L<sub>4</sub> larva was offered to a group of ten mated *E. vuilleti*  
122 | females confined to an arena (diameter = 8 cm, height = 2.5 cm) containing the capsules. The  
123 | bruchid L<sub>4</sub> larvae were placed inside the capsules after removal from seeds by dissection and

124 selection by size ( $9.12 \pm 0.20$  mg). Four replicated experiments of ten ovipositing females  
125 each were performed at the same time and ovipositions were observed in a rearing chamber  
126 ( $33^{\circ}\text{C}$ :  $23^{\circ}\text{C}$ , 12L: 12D, r.h. 70%). Experiments were performed for one week during which  
127 females presented substantial egg-laying activity and did not run out of sperm in the  
128 spermatheca (Darrouzet et al., 2002). There were two four-hour exposure periods every day,  
129 and the parasitism behaviour of females was analysed at the end of each period. Every time  
130 two eggs were laid on the same host, each egg was transferred onto a different unparasitized  
131 host and deposited in a different cell in a Plexiglas sheet closed by a Plexiglas cover-slide  
132 until emergence of the parasitoid adults. Sex ratio calculations were carried out only when  
133 both eggs developed successfully. The sex ratio was measured as the proportion of females  
134 emerging from the cells in the Plexiglas sheets, i.e. the secondary sex ratio. At the end of each  
135 four-hour exposure period, entire patches were prepared with new capsules.

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

139

#### 140 *b) Sex ratio at emergence under superparasitism*

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

147

#### 148 *2) Superparasitism experiments with individual females*

149 *a) Sex ratio deposited under self-superparasitism*

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

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

156

157 *b) Sex ratio deposited under conspecific superparasitism*

158 To determine the sex of an egg laid in conspecific superparasitism, a patch of ten gelatine  
159 capsules each containing one *C. maculatus* L<sub>4</sub> larva was offered in the morning to ten virgin  
160 *E. vuilleti* females confined to an arena. Nine replicated experiments were performed.  
161 Capsules with parasitized hosts were isolated at midday. In the afternoon, five of these  
162 capsules, each containing one parasitized host, were offered to an isolated mated female (n =  
163 15 replicates). When a mated female laid one egg, the two eggs on the same host were  
164 transferred onto a different host and deposited in a Plexiglas sheet to obtain the sex of the two  
165 parasitoid adults. Sex ratio calculations were carried out only when both eggs laid on the same  
166 host developed successfully.

167

168 *Calculations and statistical analysis*

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



174 a quarter to have two male eggs. In superparasitism, the sex ratio of surviving adults was 0.51  
175 (see Results). Thus, if sex were allocated to eggs at random, but with a mean proportion of  
176 0.51 females, when two eggs are laid per host we would expect proportions of 0.26 hosts  
177 ( $0.51 \times 0.51$ ) to bear two females, 0.24 hosts ( $0.49 \times 0.49$ ) to bear two males and 0.50 hosts  
178 ( $[0.49 \times 0.51] + [0.51 \times 0.49]$ ) to bear a mixed-sex brood. These proportions were multiplied  
179 by our sample size to give the expected numbers. This mean sex-ratio gave us an expected  
180 binomial frequency distribution of sex ratio. This binomial distribution was then compared  
181 with the experimental data (Figure 1). Experimental data were taken into account only when  
182 the two eggs laid together and then transferred onto a different host developed successfully.

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

189

## 190 **Results**

### 191 *Sex allocation by groups of ten females.*

192 *E. vuilleti* females in groups laid two eggs on the same host with an unbiased sex ratio  $SR =$   
193  $0.57 \pm 0.05$  ( $n = 10$  groups of ten females which laid 204 pairs of eggs;  $\chi^2 = 13.38$ , d.f. = 9,  $P$   
194  $= 0.14$ ). These females preferentially laid two eggs of the same sex on the same host (61.76%  
195 of clutches), and in 37.75% of cases two female eggs. These observed clutch percentages in a  
196 female population differed from those expected from a binomial distribution ( $\chi^2 = 7.6$ , d.f. =  
197 2,  $P < 0.025$ ) (Figure 1).

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

200

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

202 After larval fights, the sex ratio of surviving parasitoids was unbiased (SR =  $0.51 \pm 0.07$ ; n =  
203 4 patches of 10 females which laid 138 pairs of eggs;  $\chi^2 = 2.57$ , d.f. = 3, P = 0.27) and was not  
204 different from the SR of eggs before the larval fights ( $0.51 \pm 0.07$  vs.  $0.57 \pm 0.05$ , Mann-  
205 Whitney U-test: Z=0.51, P=0.69).

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

207

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

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

214 22.87% of eggs laid in parasitism failed to develop when ovipositing females were in groups,  
215 and 16.45% in the case of isolated females.

216

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

218 Isolated *E. vuilleti* females produced a female-biased sex ratio in self-superparasitism (SR =  
219  $0.68 \pm 0.05$ , n = 16 females which laid 154 pairs of eggs;  $\chi^2 = 47.29$ , d.f. = 15, P < 0.005).  
220 Females laid one egg with an unbiased sex ratio on a host already parasitized by a conspecific  
221 virgin female (SR =  $0.51 \pm 0.06$ , n = 15 females which superparasitized 142 hosts;  $\chi^2 = 15.04$ ,

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

224 In 6.24% of superparasitism cases, both eggs of the same pair failed to develop, and in  
225 37.55% of cases, one egg failed to develop.

226

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

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

233

## 234 **Discussion**

235 When in groups, *E. vuilleti* females produced an unbiased offspring sex ratio from  
236 parasitized and superparasitized hosts. Isolated females produced a female-biased sex ratio in  
237 parasitism and self-superparasitism, but an unbiased offspring sex ratio in conspecific  
238 superparasitism. These patterns of sex ratio behaviour in this species are consistent with the  
239 predictions of the LMC model (Hamilton, 1967; Werren, 1980; Shuker et al., 2005). In  
240 different parasitoid species, females increase the proportion of their sons when other females  
241 are present (King, 1987, 1993) with a predicted offspring sex ratio approaching equality (SR  
242 = 0.5). A conspecific egg on a host could also inform an isolated ovipositing female of the  
243 presence of putative conspecific females even if she never encounters them. In this situation, a  
244 female can alter her sex allocation as predicted by LMC (Shuker and West, 2004; Shuker et  
245 al., 2006). *E. vuilleti* females deposited a sex ratio in situation of conspecific superparasitism  
246 that differed from one predicted under a binomial distribution. The sex ratio deposited under

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

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

260 Superparasitism has often been linked to a competitive superiority of one sex (King,  
261 1987). This phenomenon tends to favour the sex which is the better larval competitor via a  
262 biased sex ratio (Suzuki et al., 1984; van Dijken and Waage, 1987). In *E. vuilleti*, we cannot  
263 determine morphologically which sex is the better competitor as we are unable to sex  
264 individuals in the early larval stages, i.e. at moments of larval fights, no non-invasive  
265 technique being available to sex each individual before larval fights. However, it is possible to  
266 sex individuals after larval fights by determining ploidy levels, either by counting the  
267 chromosomes (Ueno and Tanaka, 1997) or by flow cytometry (Aron et al., 2003). These  
268 ploidy techniques were not used in the present work and we simply analysed the sex of  
269 survivors at their adult stage. However, it is possible to calculate the putative sex ratios of the  
270 surviving adults according to which sex is the better competitor (Darrouzet et al., 2003). After  
271 analyzing results of egg-transfer experiments after oviposition by random females from a

272 group (figure 1), if female larvae are the better competitors, the sex ratio of surviving  
273 offspring will be 0.76; if males are the better competitors, the sex ratio will be 0.38; and if  
274 male and female have equal chances of winning the competition, the sex ratio will be 0.57.  
275 The sex ratio of surviving adults in superparasitism experiments without egg transfer was  
276 0.51, suggesting that neither males nor females have competitive superiority. This result  
277 differs from those obtained by Darrouzet et al. (2003) in self-superparasitism conditions and  
278 from which the authors suggested that females were the better competitors. The difference  
279 observed in this species about which sex is the better competitor could be linked to the genetic  
280 origin of the competitors. When there are mixed-sex clutches in self-superparasitism, larval  
281 fights take place between siblings, whereas in superparasitism by a group of females, these  
282 fights probably occur between two unrelated larvae. In this case, it is possible that during  
283 conspecific fights between the two sexes, some larval females are not good competitors  
284 (because of a delay in hatching or a large difference in size for example) and female eggs are  
285 thus eliminated by larval males.

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

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

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

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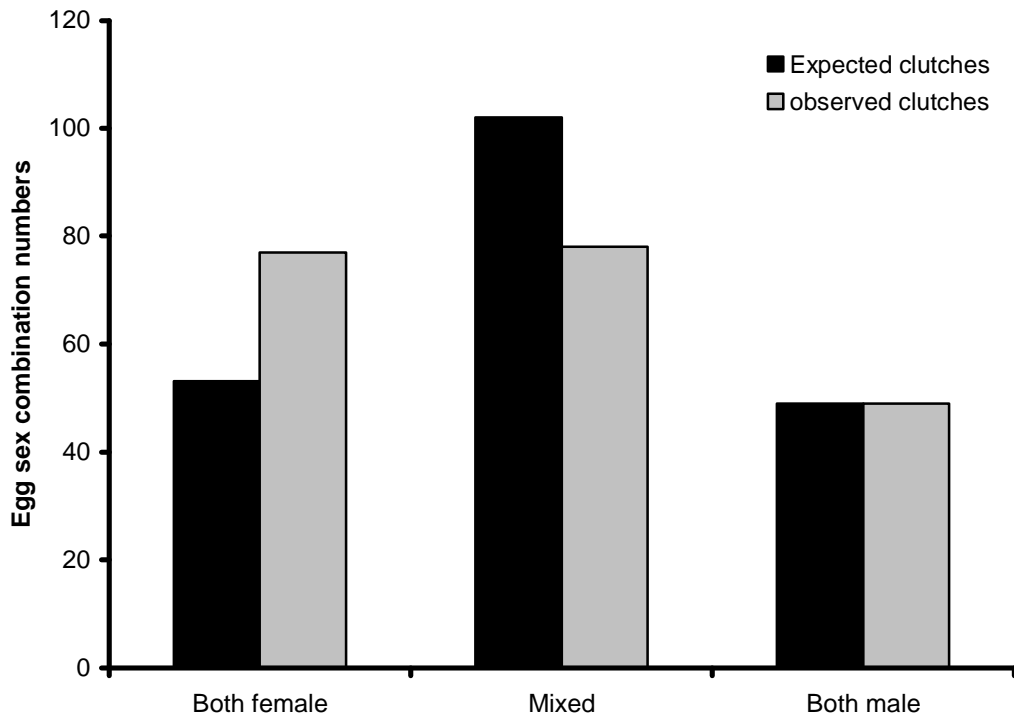
405 **Figure 1** Sex allocation decisions of four groups composed of ten mated *Eupelmus vuilleti*  
406 females laying two eggs per host in superparasitism (n = 204 pairs of eggs). Histograms in  
407 black indicate the expected clutches in a binomial sex ratio distribution = 0.51 (see Results),  
408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

409

410 **Figure 2** Comparison of egg-sex combinations of two eggs laid in superparasitism by a group  
411 or by isolated *Eupelmus vuilleti* females. Histograms in black indicate the sex allocation  
412 decisions of 16 isolated mated females laying two eggs per host in self-superparasitism (n =  
413 141 pairs of eggs) and histograms in grey the sex allocation decisions of four groups  
414 composed of ten mated females laying two eggs per host in superparasitism (n = 204 pairs of  
415 eggs).

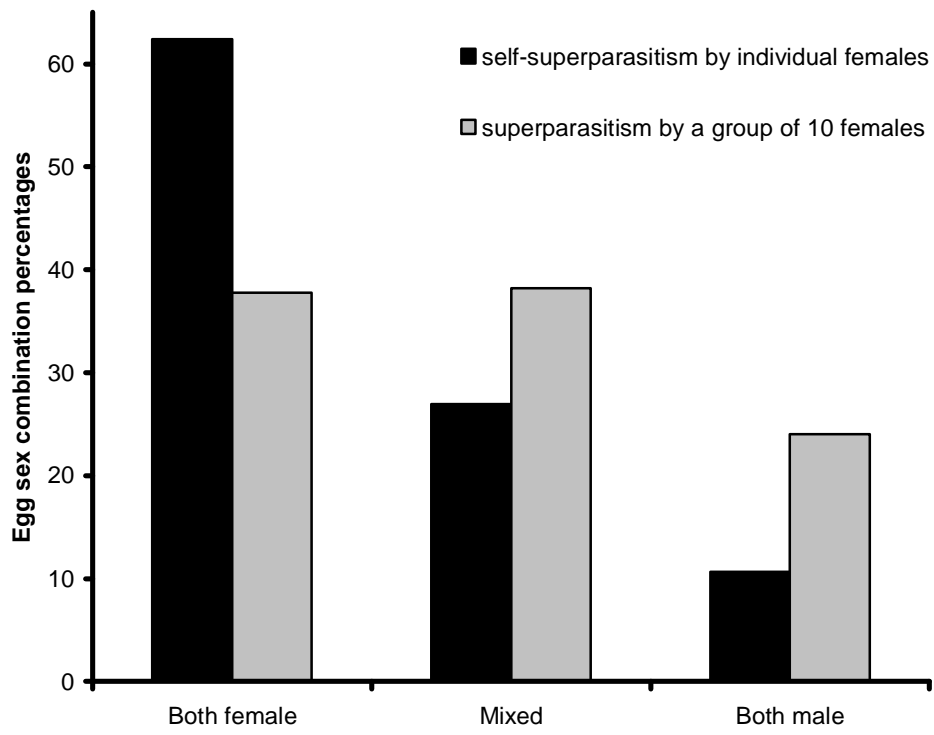
416 Figure 1:

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419 Figure 2:



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