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1 **No evidence for contribution of sexually monomorphic wing pigmentation pattern**  
2 **to mate choice in *Drosophila guttifera***

3

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10 **Key words:** Mating probability, sexually monomorphic ornament, polka-dotted pattern,  
11 fruit fly, behaviour

12

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14 **Abstract**

15           In many animal groups, sexually dimorphic ornaments are thought to evolve by  
16 intraspecific competition or mate choice. Some researchers pointed out that sexually  
17 monomorphic ornaments could also evolve by mate choice by both sexes or either sex.  
18 Many species of fruit fly have sexually monomorphic wing pigmentation. However,  
19 involvement of their sexually monomorphic ornaments in mate choice has not been tested.  
20 We aimed to examine whether the sexually monomorphic polka-dotted pattern on wings  
21 of *Drosophila guttifer* contributes to mate choice. Because *D. guttifer* does not mate in  
22 the dark condition at all and no courtship sound has been observed, some visual  
23 information is likely to be used in mating behaviour. We compared the number of matings  
24 between individuals with and without wings, and found that presence of wings influenced  
25 mating behaviour in both sexes. We then compared the number of matings between  
26 individuals bearing replaced wings, one group bearing conspecific *D. guttifer* wings and  
27 another group bearing heterospecific *D. melanogaster* wings with no pigmentation  
28 pattern. An effect of conspecific/heterospecific wings was only detected in mate choice  
29 by females. Comparison between wild-type and black-painted wings revealed no  
30 evidence of a contribution of wing pigmentation pattern to mate choice in either sex.

31 **Introduction**

32 Evolution of sexually dimorphic ornaments has been explained in the theoretical  
33 framework of sexual selection, such as Fisher's runaway process and the handicap  
34 principle (Fisher, 1915; Zahavi, 1975). In many animal groups, sexually dimorphic  
35 ornaments were suggested to evolve by intrasexual competition and mate choice (Petrie,  
36 Halliday, & Sanders, 1991; Petrie & Halliday, 1994; Andersson, 1982; Ryan, 1985; Theis,  
37 Salzburger, & Egger, 2012). Some researchers argued that sexually monomorphic  
38 ornaments could evolve before sexually dimorphic ornaments under the evolutionary  
39 restriction of sexual dimorphism by genetic correlation or constraint between the two  
40 sexes, and that dimorphism evolves by mutations enabling circumvention of the genetic  
41 constraint on a long timescale (Lande, 1980). The details of genetic constraints are now  
42 understood according to a concept of intralocus sexual conflict (Bonduriansky &  
43 Chenoweth, 2009). For example, king penguins, *Aptenodytes patagonicus*, have sexually  
44 monomorphic ornaments, but female mate choice was observed to be stronger than male  
45 mate choice (Pincemy, Dobson, & Jouventin, 2009). This suggests that sexually  
46 monomorphic ornaments could evolve by a mate choice by either sex. As a counter  
47 explanation, the same traits could be involved in mate choices of both sexes that result in  
48 evolution of a sexually monomorphic ornament (Kraaijeveld, Kraaijeveld-Smit, &  
49 Komdeur, 2007).

50 In *Drosophila* (fruit flies), there are species with various pigmentation patterns  
51 on wings (Koshikawa, 2020). Many species of *Drosophila* are known to have male-  
52 specific black pigmentation on the anterior-distal part of wings (Kopp & True, 2002).  
53 Males of these species appear to display their wings in front of females (Prud'homme et  
54 al., 2006). However, an effect of wing pigmentation on mate choice was not always  
55 observed. Using three species with sexually dimorphic black spots on male wings  
56 (*Drosophila suzukii*, *D. biarmipes* and *D. subpulchrella*), Roy and Gleason (2019)  
57 examined whether females prefer males with spots or males without spots (that were  
58 made by CO<sub>2</sub> anaesthesia after eclosion). An effect of spots on mate preference was not  
59 detected in their study. Fuyama (1979) revealed that males without spots as a result of  
60 amputation showed lower mating frequency than intact males in *D. suzukii* when females  
61 were kept in constant light to make them less accepting of mating. In *D. biarmipes*, which  
62 has natural polymorphism of wing pigmentation, males with pigmentation on their wings  
63 showed greater mating success than males without pigmentation (Hegde, Chethan, &  
64 Krishna, 2005; Singh & Chatterjee, 1987). The effect of pigmentation was dependent on  
65 environmental conditions (Parkash, Lambhod, & Singh, 2013).

66 Despite having sexually monomorphic pigmentation on their wings, some fruit

67 fly males display their wings in front of females during their courtship. For example, in  
68 *Idiomya grimshawi* (synonym of *Drosophila grimshawi*), adults aggregate in leks and  
69 males display with their elaborately pigmented wings (Spieth, 1966; Edwards, Doescher,  
70 Kaneshiro, & Yamamoto, 2007). However, the function of this sexually monomorphic  
71 pigmentation during courtship has not been studied experimentally. Many species of fruit  
72 flies are known to have sexually monomorphic wing pigmentations (Patterson, 1943;  
73 Miller, Marshall, & Grimaldi, 2017; Werner, Steenwinkel, & Jaenike, 2020; Dufour,  
74 Koshikawa, & Finet, 2020), but no functional testing of these monomorphic traits has  
75 been reported.

76 *Drosophila guttifer* has been used as a research model for elucidating  
77 pigmentation pattern formation (Werner, Koshikawa, Williams, & Carroll, 2010;  
78 Koshikawa et al., 2015; Koshikawa, Fukutomi, & Matsumoto, 2017; Fukutomi,  
79 Matsumoto, Agata, Funayama, & Koshikawa, 2017; Dion et al. 2020; Shittu, Steenwinkel,  
80 Koshikawa, & Werner 2020). This species has a sexually monomorphic polka-dotted  
81 pattern on its wings, but the function of the pattern has not been explored. Because *D.*  
82 *guttifer* does not mate at all in a dark condition, visual information is likely to be used  
83 in mating (Grossfield, 1966). In addition, courtship sound, such as wing vibration, was  
84 not observed in this species (Spieth, 1952; Grossfield, 1977; Wen & Li, 2011).

85 The purpose of this study was to clarify whether the sexually monomorphic  
86 pigmentation pattern in *D. guttifer* contributes to mate choice. We examined the effect  
87 of the presence of wings and the polka-dotted pattern on wings on mate choice of both  
88 sexes by conducting mating experiments with cutting and replacing of wings.

89

## 90 **Materials and methods**

### 91 Fly

92 *Drosophila guttifer* inhabits North America and is related to the *quinaria* group  
93 (Chialvo, White, Reed, & Dyer, 2019; Izumitani, Kusaka, Koshikawa, Toda, & Katoh,  
94 2016). In this study, adults of the wild type *D. guttifer* (stock number 15130-1971.10,  
95 provided by the Drosophila Species Stock Center at the University of California, San  
96 Diego) kept in our laboratory were used. In addition to intact adults (“wild type”), adults  
97 whose wings were cut within 24 hours after eclosion (“no wing”) and adults whose wings  
98 were painted black 9–12 days after eclosion (“black wing”) were prepared. Also, adults  
99 whose wings were replaced by *D. guttifer* wings (“*guttifer* wing”), wings with an  
100 incomplete pigmentation pattern (“incomplete pattern”), wings of *Drosophila*  
101 *melanogaster* Oregon-R (“*melanogaster* wing”), and black-painted wings of *D.*  
102 *melanogaster* (“*melanogaster* black wing”) were prepared and then maintained for 14-87

103 hours before experiments (the recipients were wild type *D. guttifera* 5-11 days after  
104 eclosion). These flies were used in mate choice experiments (Figure 1). The strain with  
105 an incomplete pigmentation pattern has a recessive mutation, but the genetic lesion of this  
106 mutation is unknown (Wataru Yamamoto, personal communication). Wings were  
107 replaced using a cyanoacrylate adhesive (Aron Alpha, Konishi Co., Ltd., Japan). To  
108 produce “black wing” flies, flies’ entire wings were painted with a black ink marker  
109 (Mckie, Zebra Co., Ltd., Japan). All individuals were reared with standard  
110 cornmeal/sugar/yeast/agar food (Fukutomi, Matsumoto, Funayama, & Koshikawa, 2018)  
111 under a photoperiod of 12:12 (light:dark) at 25°C.

112

### 113 Sexual maturity after eclosion

114 To prepare virgin individuals for mating experiments, we had to confirm that  
115 adults were not sexually mature within 24 hours after eclosion. Firstly, males and females  
116 were separated into different vials within 4 hours after eclosion. Some vials were kept 7–  
117 10 days after eclosion. Four females 7–10 days after eclosion and four males 4 hours after  
118 eclosion were introduced per one vial (n=5). Likewise, four males 7–10 days after  
119 eclosion and four females 4 hours after eclosion were introduced per one vial (n=6). In a  
120 control group, four females and four males 7–10 days after eclosion were introduced per  
121 one vial (n=4). These three groups of vials were kept for 24 hours, and then adults in the  
122 vials were removed. The number of larvae in the vials was counted 5–7 days after adults  
123 had been removed.

124 For mate choice experiments, adult males and females within 24 hours after  
125 eclosion were collected and kept in groups of 1–20 adults of the same sex. Mate choice  
126 experiments were conducted 5–13 days after eclosion.

127

### 128 Recording of mating behaviour

129 All mate choice experiments were conducted in chambers made of acrylic resin  
130 (10 mm in diameter and 5 mm in height). A small piece of fly food was placed in each  
131 chamber to prevent drying of flies during experiments. The chambers were put on a piece  
132 of white paper, and then adults used in experiments were introduced into the chambers.  
133 The chambers were then illuminated with a halogen lamp (PICL-NEX, Nippon P·I, Co.,  
134 Ltd., Japan) and recorded with a web camera (C615n, Logicool Co., Ltd., Japan) or a  
135 digital camera (HDR-PJ590V, Sony, Japan) continuously for 3 hours. The start time of  
136 the recording was between 90 and 150 minutes after the light phase of the original rearing  
137 condition.

138

139 Preference for wings

140 In a male mate choice experiment (Table 1; competition experiment with one  
141 male and two females per one chamber) to examine the preference for wings, we observed  
142 whether males mated with “wild type” females or “no wing” females. The kinds of mating  
143 were classified into four types—the first mating between one male and one “wild type”  
144 female, the second mating between them, the first mating between one male and one “no  
145 wing” female, and the second mating between them. As another index of the preference  
146 for wings, we observed whether males courted females and which female was courted  
147 first. Also, we measured courtship latency and courtship duration and compared them.  
148 We defined two types of courtship latencies and two types of courtship durations. Latency  
149 1: the length of time (min) from the start of recording to the first courtship. Latency 2:  
150 the length of time from the start of recording to the first successful courtship followed by  
151 mating. Duration 1: the length of the first courtship. Duration 2: the length of the first  
152 successful courtship followed by mating. Latency 1 and Duration 1 mostly reflect only  
153 male’s preference. Latency 2 and Duration 2 reflect females’ eagerness to mate as well as  
154 male’s preference, since they depend on the length of time until females accept to be  
155 courted and mate. Latency 1 could be the same as Latency 2, and Duration 1 could be the  
156 same as Duration 2, when successful courtship followed by mating was immediately  
157 observed after the start of recording.

158 In a female choice experiment (Table 1; competition experiment with one female  
159 and two males per one chamber) to examine the preference for wings, we observed  
160 whether females mated with “wild type” males or “no wing” males. In this experiment,  
161 the difference between the number of matings achieved by two males may be influenced  
162 by female preference and also males’ eagerness to mate. To estimate males’ eagerness to  
163 mate, we observed whether males courted females and which male courted first. Also, we  
164 measured the courtship latency (Latency 1: the length of time from the start of recording  
165 to the first courtship).

166

167 Preference for conspecific wings

168 In a male choice experiment (Table 1; competition experiment) to examine the  
169 preference for the polka-dotted pattern, we observed whether males mated with “*guttifera*  
170 wing” females or “*melanogaster wing*” females. The kinds of mating were classified into  
171 four types—the first mating between one male and one “*guttifera wing*” female, the  
172 second mating between them, the first mating between one male and the one  
173 “*melanogaster wing*” female, and the second mating between them. As another index of  
174 the preference for the polka-dotted pattern, we observed whether males courted females

175 and which female was courted first. Also, courtship latencies (Latency 1 and 2) and  
176 courtship durations (Duration 1 and 2) were measured.

177 In a female choice experiment (Table 1; competition experiment) to examine the  
178 preference for the polka-dotted pattern, we observed whether females mated with  
179 “*guttifera* wing” males or “*melanogaster* wing” males. To estimate males’ eagerness to  
180 mate, we observed whether males courted females and which male courted first. Also, we  
181 measured the courtship latency (Latency 1).

182

183 Preference for the polka-dotted pattern

184 In a female choice experiment (Table 1; competition experiment) to examine the  
185 preference for the precise number of polka dots, we observed whether females mated with  
186 “*guttifera* wing” males or “incomplete pattern” males. Also, to examine the preference  
187 for dark colour of the entire wings, we observed whether females mated with “wild type”  
188 males or “black wing” males. Finally, we observed whether females mated with  
189 “*melanogaster* wing” males or “*melanogaster* black wing” males. In these three  
190 experiments, to estimate males’ eagerness to mate, we observed whether males courted  
191 females and which male courted first. Also, we measured the courtship latency (Latency  
192 1).

193

194 Statistical analysis

195 All statistical analyses were performed using R ver. 3.5.2 (R Core Team, 2018).  
196 In both male and female mate choice experiments, GLMs were used to investigate the  
197 influence of the mating combination between the sexes, male age, female age and hours  
198 after females had received replaced wings (if the experiment included wing replacement)  
199 on the occurrence of mating. We included ages and hours after wing replacements in this  
200 analysis, because ages and CO<sub>2</sub> anaesthesia are known to affect the behavior of some  
201 *Drosophila* species (Avent, Price & Wedell, 2008; Colinet & Renault, 2012; Verspoor,  
202 Cuss & Price, 2015). The occurrence of mating was treated as a response variable  
203 assuming a binomial distribution. Mating combination, male age, female age and hours  
204 after females had received replaced wings were treated as explanatory variables. As a link  
205 function, logit function was used. In addition to GLMs, chi-squared tests were performed  
206 on the mating combination between the sexes without variables of ages. For the three  
207 experiments to test the preference for the polka-dotted pattern, Bonferroni correction with  
208 chi-squared test was performed to counteract the problem of multiple similar experiments  
209 (Wright, 1992). When sample size was less than 40, Yates correction with the chi-squared  
210 test was performed (Campbell, 2007). For comparison of courtship latencies and



211 courtship durations, we used the Student's *t*-test. To determine which type of female was  
212 courted first and which type of male courted first, we used the exact binomial test.

213

## 214 **Results**

215 The courtship behaviour of *D. guttifera*

216 The courtship behaviour of both males and females was observed. Firstly, a male  
217 approached a female from behind, the side or the front. Next, the male chased the female  
218 if the female ran away from the male. Then, the male tapped female body parts (mostly  
219 top of the wing or head) with its foreleg. Next, the male extended its proboscis and licked  
220 the female's ovipositor. Following this behaviour, the male intensely vibrated its  
221 proboscis toward the female. No wing motion (extension, vibration, scissoring, flicking  
222 or rotation) of the male was observed. A receptive female spread its wings widely and  
223 then the male mounted the female. This courtship behaviour seems to be identical to those  
224 observed in previous studies (Spieth, 1952; Grossfield, 1966; Grossfield, 1977).

225

226 Sexual immaturity at 24 hours after eclosion

227 Four males 7–10 days after eclosion and four females 4 hours after eclosion were  
228 introduced per vial and were removed from the vial after 24 hours. After 5–7 days, no  
229 larvae were observed in the vials (n=5). Also, no larvae were observed in vials into which  
230 four females 7–10 days after eclosion and four males 4 hours after eclosion were  
231 introduced and then removed (n=6). In contrast, the number of larvae was  $15.8 \pm 11.0$   
232 (mean  $\pm$  SD [standard deviation], n=4) in vials into which four males and four females  
233 (all 7–10 days after eclosion) were introduced and then removed.

234 We concluded that neither males nor females become sexually mature within 24  
235 hours after eclosion. Therefore, in the following experiments, the adults within 24 hours  
236 after eclosion were collected as virgins. We kept virgins for more than 5 days in groups  
237 and used them in mate choice experiments.

238

239 Preference for wings

240 For male choice experiments, we conducted 51 experiments to examine  
241 preference for wings (Figure 2a). Some males mated with both of the two females. Such  
242 matings were recorded separately as the first mating and the second mating. Males never  
243 mated twice with the same female in our observations. In the first matings, males mated  
244 with "wild type" females 25 times and with "no wing" females 10 times. In the second  
245 matings, males mated with "wild type" females four times and with "no wing" females  
246 11 times. Thus, at the first mating, males mated with "wild type" at higher rate than with

247 “no wing”, and at the second mating males mated with “no wing” females at higher rate  
248 than with “wild type” females (GLM, estimate=-2.7865,  $p=0.000682$ ; chi-squared test,  
249  $p=0.0006539$ ). Other explanatory variables were not statistically significant (GLM,  
250  $p>0.05$ ).

251 We observed courtships as another index of preference for wings in these 51  
252 experiments. Males courted females in 47 experiments in total. Among 15 experiments in  
253 which the male mated with both females, we observed courting both females in all 15  
254 experiments. Among 20 experiments in which the male mated with one female, we  
255 observed courting both females in 16 experiments and courting one female in four  
256 experiments. Among 16 experiments in which the male did not mate, we observed  
257 courting both females in eight experiments, courting one female in four experiments, and  
258 no courting in four experiments. Males courted first “wild type” females 29 times and  
259 “no wing” females 18 times, which were not significantly different (exact binomial test,  
260  $p=0.1439$ ). Courtship latencies were not significantly different between courtships with  
261 “wild type” females (Latency 1,  $29.5 \pm 36.0$  min [mean  $\pm$  SD]; Latency 2,  $43.3 \pm 35.5$   
262 min) and courtships with “no wing” females (Latency 1,  $25.9 \pm 25.8$  min; Latency 2,  $62.9$   
263  $\pm 47.4$  min) ( $t$ -test,  $p=0.6054$  and  $p=0.1067$ ). Courtship durations were significantly  
264 different between courtships with “wild type” females (Duration 1,  $0.49 \pm 0.69$  min;  
265 Duration 2,  $0.69 \pm 0.89$  min) and courtships with “no wing” females (Duration 1,  $1.19 \pm$   
266  $2.18$  min; Duration 2,  $2.32 \pm 2.41$  min) ( $t$ -test,  $p=0.04862$  and  $p=0.001912$ ). The  
267 significant difference of these courtship durations may indicate the difference of female’s  
268 receptive behaviour, and it could be a part of the reason why “no wing” females mated  
269 less than “wild type” females (see Discussion).

270 For female choice experiments, we conducted 51 experiments to examine  
271 preference for wings (Figure 2b). Females mated with “wild type” males 27 times and  
272 with “no wing” males 15 times. Females never mated twice with the same male in our  
273 observations. Females mated with “wild type” males more frequently than with “no wing”  
274 males (GLM, estimate=1.0313,  $p=0.0158$ ; chi-squared test,  $p=0.01577$ ). Other  
275 explanatory variables were not statistically significant (GLM,  $p>0.05$ ).

276 We observed courtships to estimate males’ eagerness to mate in these 51  
277 experiments. Males courted females in all 51 experiments. Among 42 experiments with  
278 successful matings, both males courted in 38 experiments and one male courted in four  
279 experiments. Among nine experiments without matings, both males courted in eight  
280 experiments and one male courted in one experiment. Females were courted first by “wild  
281 type” males in 22 experiments and by “no wing” males in 29 experiments, which were  
282 not significantly different (exact binomial test,  $p=0.4011$ ). The courtship latency (Latency

283 1) of “wild type” males ( $15.8 \pm 21.6$  min) and that of “no wing” males ( $18.0 \pm 22.0$  min)  
284 were not significantly different ( $t$ -test,  $p=0.6192$ ). Therefore, there was no evidence of a  
285 difference in males’ eagerness to mate. In summary, females were found to prefer males  
286 with wings to males without wings.

287

#### 288 Preference for conspecific wings

289 For male choice experiments, we conducted 50 experiments to examine the  
290 preference for the polka-dotted pattern (Figure 3a). In the first matings, males mated with  
291 “*guttifera* wing” females 19 times and “*melanogaster* wing” females 14 times. In the  
292 second matings, males mated with “*guttifera* wing” females eight times and *D.*  
293 *melanogaster* wing females nine times. No significant difference was seen between the  
294 rate of mating of males with “*guttifera* wing” wing females, and that of males with  
295 “*melanogaster* wing” females (GLM, estimate=0.24864,  $p=0.544$ ; chi-squared test,  
296  $p=0.4237$ ). Other explanatory variables were also not statistically significant (GLM,  
297  $p>0.05$ ).

298 We observed courtships as another index of preference for the polka-dotted  
299 pattern in these 50 experiments. Males courted females in 48 experiments in total. Among  
300 17 experiments in which the male mated with both females, we observed courting both  
301 females in all 17 experiments. Among 16 experiments in which the male mated with one  
302 female, we observed courting both females in 13 experiments and courting one female in  
303 three experiments. Among 17 experiments in which the male did not mate, we observed  
304 courting both females in 10 experiments, courting one female in five experiments, and no  
305 courting in two experiments. Males courted first “*guttifera* wing” females in 28  
306 experiments and “*melanogaster* wing” females in 20 experiments, which were not  
307 significantly different (exact binomial test,  $p=0.3123$ ). Courtship latencies were not  
308 significantly different between courtships with “*guttifera* wing” females (Latency 1,  $16.1$   
309  $\pm 23.9$  min; Latency 2,  $41.7 \pm 41.6$  min) and courtships with “*melanogaster* wing”  
310 females (Latency 1,  $14.7 \pm 17.6$  min; Latency 2,  $35.3 \pm 27.6$  min) ( $t$ -test,  $p=0.7698$  and  
311  $p=0.5498$ ). Courtship durations were not significantly different between courtships with  
312 “*guttifera* wing” females (Duration 1,  $1.27 \pm 3.51$  min; Duration 2,  $1.39 \pm 1.86$  min) and  
313 courtships with “*melanogaster* wings” females (Duration 1,  $0.61 \pm 0.76$  min; Duration 2,  
314  $1.30 \pm 0.87$  min) ( $t$ -test,  $p=0.2351$  and  $p=0.8428$ ).

315 For female choice experiments, we conducted 49 experiments to examine the  
316 preference for the polka-dotted *D. guttifera* wings (Figure 3b). Females mated with  
317 “*guttifera* wing” males 22 times and “*melanogaster* wing” males 12 times. Females mated  
318 with “*guttifera* wing” males significantly more frequently than with “*melanogaster* wing”

319 males (GLM, estimate=0.94335,  $p=0.0361$ ; chi-squared test,  $p=0.03382$ ). Other  
320 explanatory variables were not statistically significant (GLM,  $p>0.05$ ).

321 We observed courtships to estimate males' eagerness to mate in these 49  
322 experiments. Males courted females in 48 experiments. Among 34 experiments with  
323 successful matings, both males courted in 30 experiments and one male courted in four  
324 experiments. Among 15 experiments without matings, both males courted in 12  
325 experiments, one male courted in two experiments, and no courting was observed in one  
326 experiment. Females were courted first by "*guttifera* wing" males in 23 experiments and  
327 "*melanogaster* wing" males in 25 experiments, which were not significantly different  
328 (exact binomial test,  $p=0.8854$ ). The courtship latency (Latency 1) of "*guttifera* wing"  
329 males ( $12.8 \pm 24.0$  min) and that of "*melanogaster* wing" males ( $11.7 \pm 25.9$  min) were  
330 not significantly different ( $t$ -test,  $p=0.8351$ ). Therefore, there was no evidence of the  
331 difference of males' eagerness to mate. In summary, females were found to prefer  
332 "*guttifera* wing" males to "*melanogaster* wing" males. In these experiments, however,  
333 we could not distinguish whether females preferred conspecific wings or the polka-dotted  
334 pattern. For this reason, we performed additional experiments using conspecific but  
335 differently patterned wings.

336

#### 337 Preference for polka-dotted pattern

338 In these additional experiments, we conducted 30 experiments to examine  
339 females' preference for different pigmentation patterns (Figure 4a). Females mated with  
340 "*guttifera* wing" males eight times and "incomplete pattern" males 10 times, and these  
341 rates were not significantly different (GLM, estimate=-0.328292,  $p=0.572$ ; chi-squared  
342 test with Yates correction  $p=0.7782$ ; with Bonferroni correction,  $p=1$ ). Other explanatory  
343 variables were also not statistically significant (GLM,  $p>0.05$ ). Also, we observed  
344 courtships to estimate males' eagerness to mate in these 30 experiments. Males courted  
345 females in 29 experiments. Among 18 experiments with successful matings, both males  
346 courted in 13 experiments and one male courted in five experiments. Among 12  
347 experiments without matings, both males courted in eight experiments, one male courted  
348 in three experiments, and no courting was observed in one experiment. Females were  
349 courted first by "*guttifera* wing" males in 15 experiments and "incomplete pattern" males  
350 in 15 experiments (exact binomial test,  $p=1$ ). The courtship latency (Latency 1) of  
351 "*guttifera* wing" males ( $9.54 \pm 11.0$  min) and that of "incomplete pattern" males ( $8.49 \pm$   
352  $12.5$  min) were not significantly different ( $t$ -test,  $p=0.7525$ ). In these results, we found no  
353 evidence of a difference in males' eagerness to mate.

354 We then considered the possibility that females might prefer darkness of male

355 wings, not a particular pattern. We conducted 38 experiments to examine preference for  
356 darkness of entire wings (Figure 4b). Females mated with “wild type” males 11 times and  
357 “black wing” males 13 times, rates that were not significantly different (GLM, estimate=  
358 0.2440,  $p=0.6219$ ; chi-squared test with Yates correction,  $p=0.8051$ ; with Bonferroni  
359 correction,  $p=1$ ). Other explanatory variables were also not statistically significant (GLM,  
360  $p>0.05$ ). Males courted females in all 38 experiments. Among 24 experiments with  
361 successful matings, both males courted in all 24 experiments. Among 14 experiments  
362 without matings, both males courted in 11 experiments and one male courted in three  
363 experiments. Females were courted first by “wild type” males in 17 experiments and  
364 “black wing” males in 21 experiments, which were not significantly different (exact  
365 binomial test,  $p=0.6271$ ). The courtship latency (Latency 1) of “wild type” males ( $19.7 \pm$   
366  $29.9$  min) and that of “black wing” males ( $9.33 \pm 13.9$  min) were not significantly  
367 different ( $t$ -test,  $p=0.06082$ ). In these results, we found no evidence of a difference in  
368 males’ eagerness to mate.

369 Finally, we conducted 41 experiments to examine whether females preferentially  
370 mated with “*melanogaster wing*” males or “*melanogaster black wing*” males (Figure 4c).  
371 Females mated with “*melanogaster wing*” males 12 times and “*melanogaster black wing*”  
372 males 12 times, rates that were not different (GLM, estimate=0.008613,  $p=0.9861$ ; chi-  
373 squared test,  $p=1$ ; Bonferroni correction,  $p=1$ ). Other explanatory variables were also not  
374 statistically significant (GLM,  $p>0.05$ ). Males courted females in all 41 experiments.  
375 Among 24 experiments with successful matings, both males courted in 22 experiments  
376 and one male courted in two experiments. Among 17 experiments without matings, both  
377 males courted in 13 experiments and one male courted in four experiments. Females were  
378 courted first by “*melanogaster wing*” males in 18 experiments and “*melanogaster black*  
379 *wing*” males in 23 experiments, which were not significantly different (exact binomial  
380 test,  $p=0.5327$ ). The courtship latency (Latency 1) of “*melanogaster wing*” males ( $15.5$   
381  $\pm 27.8$  min) and that of “*melanogaster black wing*” males ( $16.1 \pm 29.3$  min) were not  
382 significantly different ( $t$ -test,  $p=0.9283$ ). In these results, we found no evidence of the  
383 difference in males’ eagerness to mate.

384 Taking these results altogether, we found that females prefer *D. guttifera* wings  
385 to *D. melanogaster* wings, but have no preference for pigmentation pattern or wing  
386 colouration. The difference between the preference for *D. guttifera* wings and *D.*  
387 *melanogaster* wings seems to be caused by some other wing characteristics, such as wing  
388 shape or smell (See Discussion).

389

390 The courtship and mating rate in *D. guttifera*

391 In two sets of male mate choice experiments, the rates of mating were 0.686  
392 (35/51 experiments in Figure 2a) and 0.660 (33/50 experiments in Figure 3a). The rates  
393 of courtship were 0.922 (47/51 experiments in Figure 2a) and 0.960 (48/50 experiments  
394 in Figure 3a). These results show that most males courted females, suggesting that  
395 matings could be largely affected by eagerness of females, rather than that of males. As  
396 most previous studies have indicated individual differences of mate preference (Bell,  
397 Hankison, & Laskowski, 2009), there could be individual differences of eagerness in  
398 females of *D. guttifera*.

399 In five sets of female choice experiments, the rates of mating were 0.824 (42/51  
400 experiments in Figure 2b), 0.694 (34/49 experiments in Figure 3b), 0.600 (18/30  
401 experiments in Figure 4a), 0.632 (24/38 experiments in Figure 4b) and 0.585 (24/41  
402 experiments in Figure 4c). The rates of courtship were 1.000 (51/51 experiments in Figure  
403 2b), 0.980 (48/49 experiments in Figure 3b), 0.967 (29/30 experiments in Figure 4a),  
404 1.000 (38/38 experiments in Figure 4b) and 1.000 (41/41 experiments in Figure 4c).  
405 Again, these results show that most males courted females, suggesting that the mating  
406 could be affected by eagerness in females, rather than that in males.

407 Except in the experiments to examine preference for wings (Figure 2b), the rates  
408 of mating were low. This could be explained by effect(s) of artificial treatments – painting  
409 wings with a black ink marker and replacing wings using a cyanoacrylate adhesive.

410 Lastly, we examined whether females mated with males that courted first, by  
411 integrating five female choice experiments. Females mated with males that courted first  
412 in 99 experiments. Females mated with males that did not court first in 44 experiments.  
413 In summary, females mated with males that courted first significantly more than with the  
414 other males (exact binomial test,  $p=4.887e-06$ ).

415

## 416 **Discussion**

### 417 Preference for wings

418 We found that females mated more with “wild type” males than “no wing” males  
419 in female mate choice experiments. In our observations, females tended to mate with the  
420 males that courted first. However, because we found no evidence that “wild type” males  
421 courted first, we conclude that mating more with “wild type” males was due to female  
422 preference for wings. Because our observations and previous studies showed that *D.*  
423 *guttifera* males do not perform courtship with obvious wing motion (Spieth, 1952;  
424 Grossfield, 1977; Wen & Li, 2011), mate choice by females does not seem to require male  
425 wing motion. Instead, females may recognize male wing smell or shape for species  
426 identification and use these features to choose mates.

427 Similarly, males might use the presence of wings to assess the quality of females.  
428 Male mate choice has been reported in multiple *Drosophila* species (Bonduriansky, 2001;  
429 Byrne & Rice, 2006). Another potential explanation for female wing function is related  
430 to females' behaviour during a courtship sequence. Grossfield (1977) observed that  
431 receptive females spread their wings, but non-receptive females repel males by  
432 decamping and kicking males. At the moment of mate acceptance, females spread their  
433 wings widely, and this motion is likely to work as a visual signal of acceptance (Spieth,  
434 1952; Grossfield, 1966). In the male mate choice experiments, we found that 1) males  
435 mated more with "wild type" females than "no wing" females, 2) courtship latencies were  
436 not significantly different, and 3) courtship durations of males towards "no wing" females  
437 were longer than those towards "wild type" females. Based on these results, we consider  
438 it possible that wing motion of females could have a function to show acceptance and  
439 increase initiation of copulation by males, and it could be a potential reason why females  
440 without wings had a lower rate of mating than intact females. Also, we found that males  
441 courted first "wild type" females in 29 experiments and "no wing" females in 18  
442 experiments. Although this difference was not statistically significant (exact binomial test,  
443  $p=0.1439$ ), we cannot rule out the possibility that males could recognize female wing  
444 shape or smell and use these features to choose mates.

445 Previous studies showed that wing damage can influence reproductive success,  
446 survival, mortality and flight performance in various insects, such as Odonata (Combes,  
447 Crall, & Mukherjee, 2010), Hymenoptera (Cartar, 1992) and Lepidoptera (Jantzen &  
448 Eisner, 2008). The present study found that both *D. guttifer* males and females mate  
449 more with intact wild-type adults than adults without wings. This suggests that wing  
450 damage could influence the preference for mates in *D. guttifer*.

451

#### 452 Preference for conspecific wings

453 In male mate choice experiments, no significant difference was seen between the  
454 number of matings of males with "*guttifer* wing" females and those of males with  
455 "*melanogaster* wing" females. But in female mate choice experiments, females were  
456 found to mate more frequently with "*guttifer* wing" males than "*melanogaster* wing"  
457 males. In our observations, females tended to mate with males that were the first to court.  
458 However, we found no evidence that "*guttifer* wing" males were the first to court.  
459 Therefore, mating more with "*guttifer* wing" males would be due to female preference  
460 for conspecific wings. Because female mate choice generally is stronger and more  
461 common than male mate choice (Bonduriansky, 2001), these results would not be  
462 surprising.

463

464 Preference for the polka-dotted pattern

465 In female mate choice experiments, females mated more with “*guttifera* wing”  
466 males than “*melanogaster* wing” males. This result suggested two possibilities—females  
467 have a preference for the polka-dotted pattern or a preference for other traits of  
468 conspecific wings. In further experiments, we did not detect females’ preference for the  
469 details of the polka-dotted pattern or for darkness of the entire wings. Also, we found no  
470 evidence of a difference in males’ eagerness to mate.

471 Taking these results together, we did not find evidence of a contribution of the  
472 sexually monomorphic polka-dotted pattern on wings to mate choice of either of the sexes  
473 in *D. guttifera*. To our knowledge, this is the first study to examine the contribution of  
474 sexually monomorphic ornaments in *Drosophila* to mate choice. Roy and Gleason (2019)  
475 also did not detect a contribution of sexually dimorphic black spots on male wings to  
476 mate choice in three *Drosophila* species. We cannot rule out, however, the possibility of  
477 a weak preference for the polka-dotted pattern, which was not statistically significant in  
478 this study, but is strong enough to be a selective pressure to fix the pattern in the  
479 population. Also, although the preference was not found in our study in laboratory  
480 conditions, we cannot exclude the possibility of the preference in a natural habitat. So far,  
481 the contribution of sexually monomorphic ornaments to mate choice is known in king  
482 penguins (Pincemy et al., 2009; Nolan et al., 2010). In the future, the question of what  
483 conditions enable sexually monomorphic ornaments to contribute to mate choice should  
484 be examined using species of various groups.

485 A contribution to mate choice was not detected in this study, and the function of  
486 the polka-dotted pattern on wings of *D. guttifera* is still unclear. Considering the  
487 extremely sophisticated gene regulation that acts to form the polka-dotted pattern  
488 (Fukutomi & Koshikawa, 2021; Fukutomi, Kondo, Toyoda, Shigenobu, & Koshikawa,  
489 2021), it is difficult to think that the pattern evolved randomly as a neutral trait without  
490 adaptive significance. Potential candidates of the function of the polka-dotted  
491 pigmentation include thermoregulation, background matching, warning colouration and  
492 species recognition. The ecological adaptations of *D. guttifera* and related species might  
493 play an important role in the evolution of the pigmentation pattern of the present form.  
494 Future research is needed to reveal what adaptive significance the polka-dotted pattern  
495 has in *D. guttifera*.

496

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503

504 Conflict of interest

505 The authors declare that they have no conflict of interest.

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668 **Figure legends**

669 **Figure 1.**

670 Flies used in mating experiments. (a) A “wild type” adult. (b) A “no wing” adult, whose  
671 wings were removed. (c) A “black wing” adult, whose wings were painted black. (d) A  
672 “*guttifera* wing” adult, whose wings were replaced with *D. guttifera* wings. (e) A  
673 “*melanogaster* wing” adult, whose wings were replaced with *D. melanogaster* wings. (f)  
674 An “incomplete pattern” adult, whose wings were replaced with wings without  
675 pigmentation spots around the campaniform sensilla. (g) A “*melanogaster* black wing”  
676 adult, whose wings were replaced with *D. melanogaster* wings painted black.

677

678 **Figure 2.**

679 Experiments to test the preference for wings. (a) Male preference for females  
680 with/without wings. The heights of the bars (sum of gray and black areas) indicate the  
681 numbers of the first mating. The black areas represent the numbers of the second mating  
682 with the other female. The gray areas represent the number of males which mated only  
683 once. White areas represent no mates. (b) Female preference for males with/without  
684 wings.

685

686 **Figure 3.**

687 Experiments to test the preference for conspecific wings. (a) Male preference for females  
688 with “*guttifera* wing” or “*melanogaster* wing”. The heights of the bars (sum of gray and  
689 black areas) indicate the numbers of the first mating. The black areas represent the  
690 numbers of the second mating with the other female. The gray areas represent the number  
691 of males which mated only once. b) Female preference for males with “*guttifera* wing”  
692 or “*melanogaster* wing”.

693

694 **Figure 4.**

695 Experiments to test female preferences for different types of wings. (a) Preference for  
696 males with “*guttifera* wing” or “incomplete pattern”. (b) Preference for males of “wild  
697 type” or with “black wing”. (c) Preference for males with “*melanogaster* wing” or  
698 “*melanogaster* black wing”.

699

700 **Table 1.**

701 General design of mate choice experiments. One adult and two adults of the other sex  
702 (Type A and B) were used in seven mate choice experiments. Each item was observed for  
703 two types of adults.

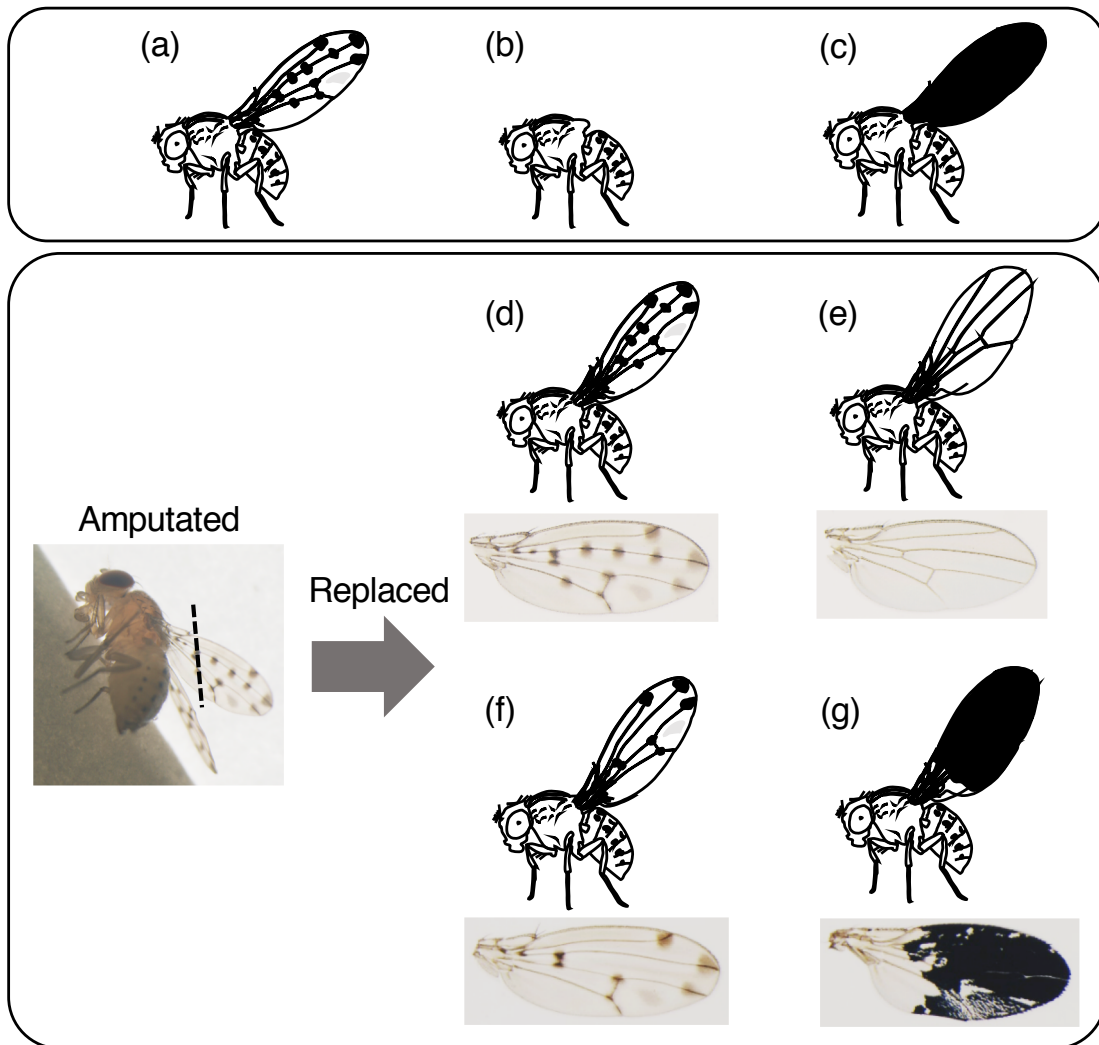


Figure 1



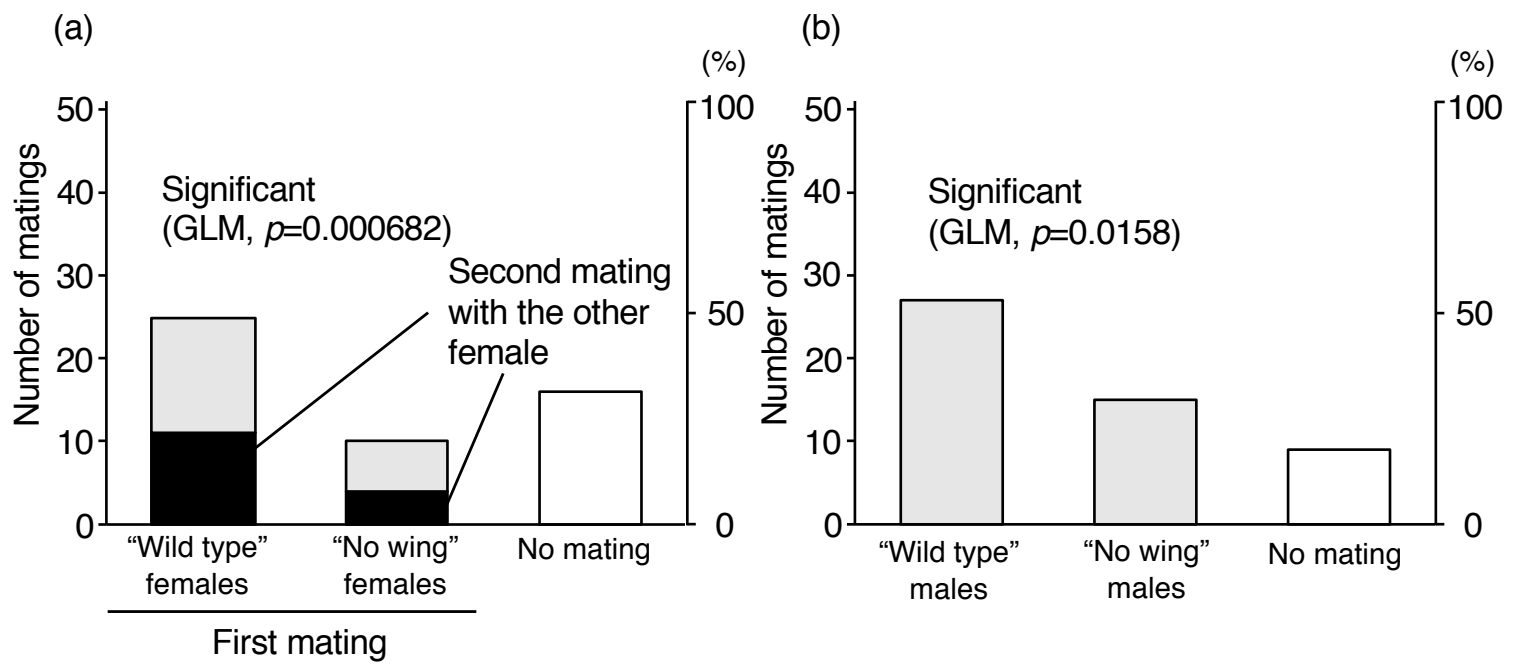


Figure 2

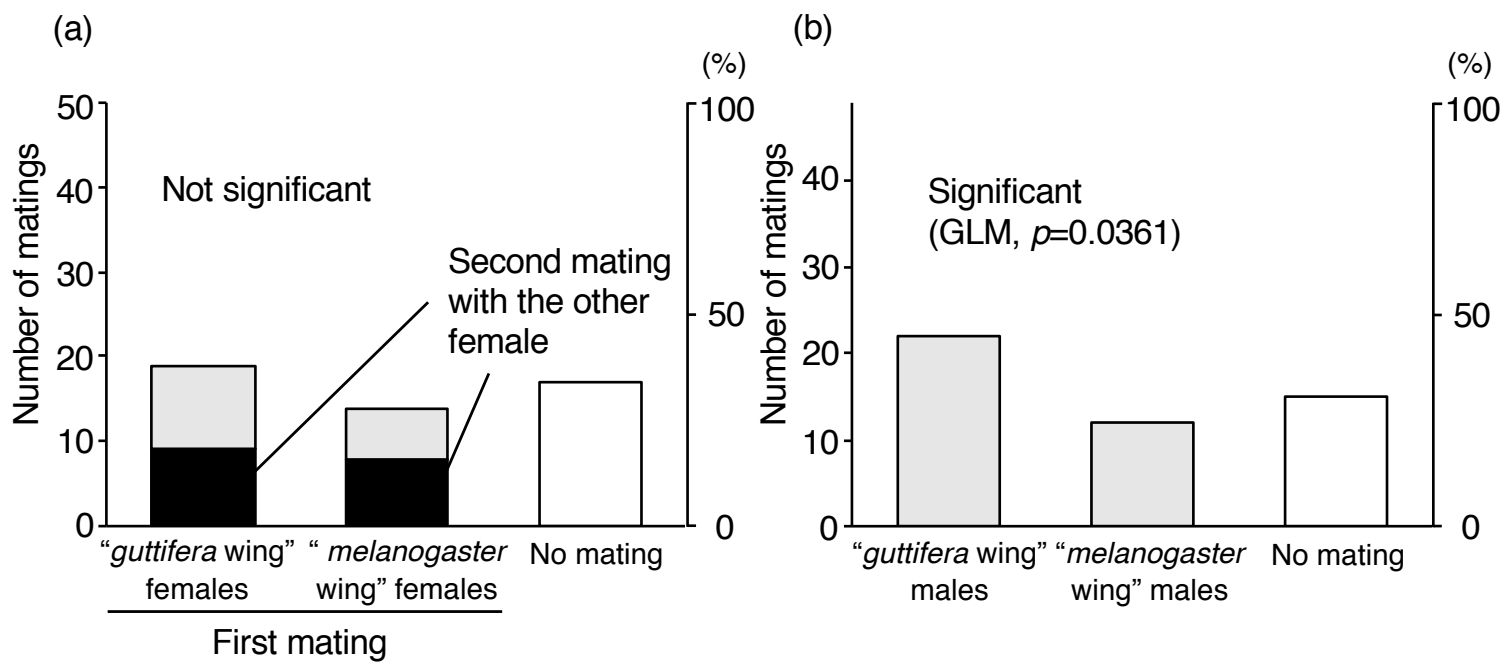


Figure 3

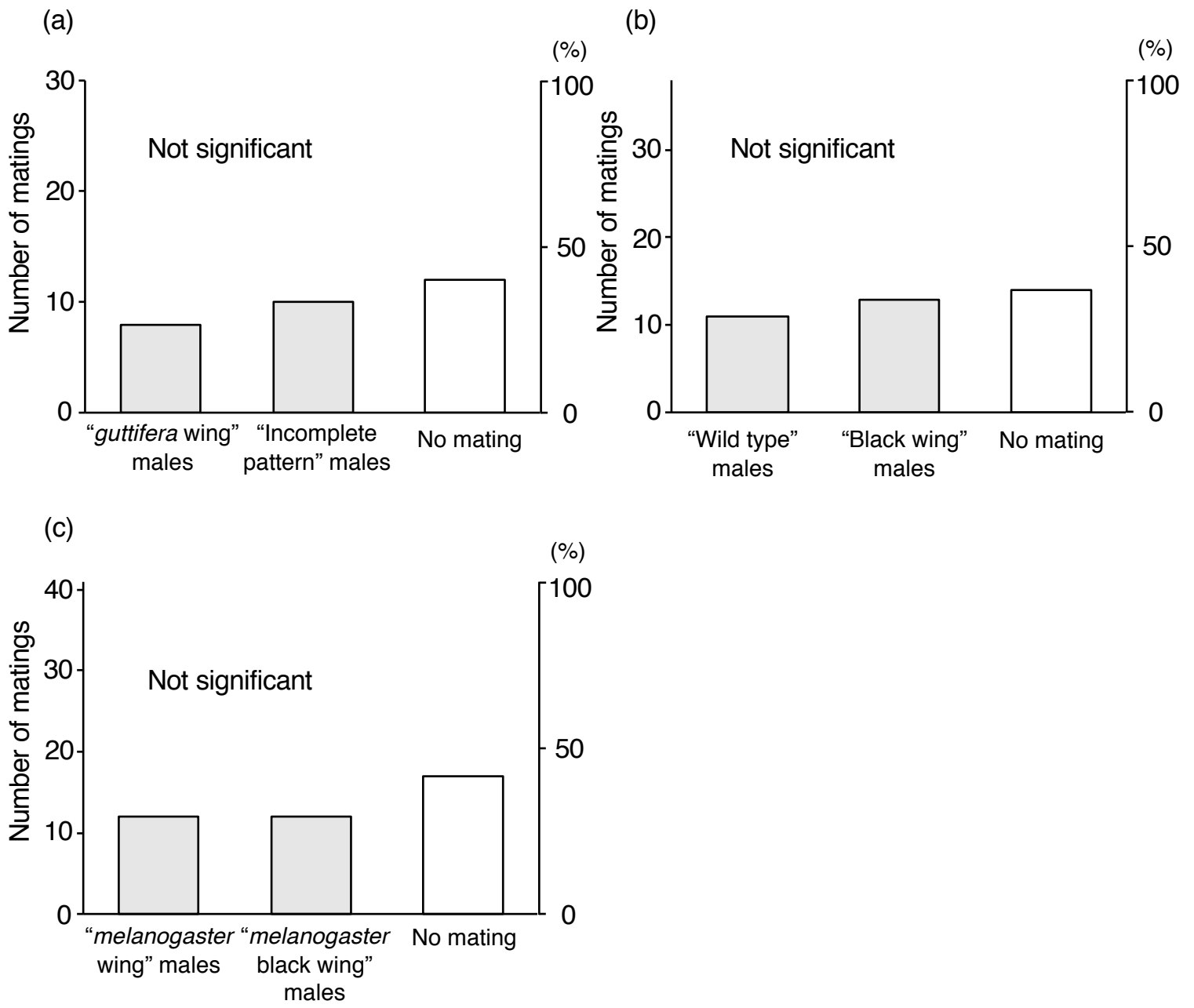


Figure 4

	Type A	Type B	Observed items
Male mate choice experiments	wild type	no wing	◆ Which type of a female mated
	<i>guttifera</i> wing	<i>melanogaster</i> wing	◆ Which type of a female was courted first
Female mate choice experiments	wild type	no wing	◆ Courtship latency and duration
	<i>guttifera</i> wing	<i>melanogaster</i> wing	
	<i>guttifera</i> wing	incomplete pattern	◆ Which type of a male mated
	wild type	black wing	◆ Which type of a male courted first
	<i>melanogaster</i> wing	<i>melanogaster</i> black wing	◆ Courtship latency

Table 1