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

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Abstract

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

Introduction

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

In *Drosophila* (fruit flies), there are species with various pigmentation patterns on wings (Koshikawa, 2020). Many species of *Drosophila* are known to have male-specific black pigmentation on the anterior-distal part of wings (Kopp & True, 2002). Males of these species appear to display their wings in front of females (Prud'homme et al., 2006). However, an effect of wing pigmentation on mate choice was not always observed. Using three species with sexually dimorphic black spots on male wings (*Drosophila suzukii*, *D. biarmipes* and *D. subpulchrella*), Roy and Gleason (2019) examined whether females prefer males with spots or males without spots (that were made by CO_2 anaesthesia after eclosion). An effect of spots on mate preference was not detected in their study. Fuyama (1979) revealed that males without spots as a result of amputation showed lower mating frequency than intact males in *D. suzukii* when females were kept in constant light to make them less accepting of mating. In *D. biarmipe*s, which has natural polymorphism of wing pigmentation, males with pigmentation on their wings showed greater mating success than males without pigmentation (Hegde, Chethan, & Krishna, 2005; Singh & Chatterjee, 1987). The effect of pigmentation was dependent on environmental conditions (Parkash, Lambhod, & Singh, 2013).

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

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

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

Materials and methods

Fly

Drosophila guttifera inhabits North America and is related to the *quinaria* group (Chialvo, White, Reed, & Dyer, 2019; Izumitani, Kusaka, Koshikawa, Toda, & Katoh, 2016). In this study, adults of the wild type *D. guttifera* (stock number 15130-1971.10, provided by the Drosophila Species Stock Center at the University of California, San Diego) kept in our laboratory were used. In addition to intact adults ("wild type"), adults whose wings were cut within 24 hours after eclosion ("no wing") and adults whose wings were painted black 9–12 days after eclosion ("black wing") were prepared. Also, adults whose wings were replaced by *D. guttifera* wings ("*guttifera* wing"), wings with an incomplete pigmentation pattern ("incomplete pattern"), wings of *Drosophila melanogaster* Oregon-R ("*melanogaster* wing"), and black-painted wings of *D. melanogaster* ("*melanogaster* black wing") were prepared and then maintained for 14-87 hours before experiments (the recipients were wild type *D. guttifera* 5-11 days after eclosion). These flies were used in mate choice experiments (Figure 1). The strain with an incomplete pigmentation pattern has a recessive mutation, but the genetic lesion of this mutation is unknown (Wataru Yamamoto, personal communication). Wings were replaced using a cyanoacrylate adhesive (Aron Alpha, Konishi Co., Ltd., Japan). To produce "black wing" flies, flies' entire wings were painted with a black ink marker (Mckie, Zebra Co., Ltd., Japan). All individuals were reared with standard cornmeal/sugar/yeast/agar food (Fukutomi, Matsumoto, Funayama, & Koshikawa, 2018) under a photoperiod of 12:12 (light:dark) at 25˚C.

Sexual maturity after eclosion

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

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

Recording of mating behaviour

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

Preference for wings

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

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

Preference for conspecific wings

In a male choice experiment (Table 1; competition experiment) to examine the preference for the polka-dotted pattern, we observed whether males mated with “*guttifera* wing” females or “*melanogaster* wing” females. The kinds of mating were classified into four types—the first mating between one male and one “*guttifera* wing” female, the second mating between them, the first mating between one male and the one “*melanogaster* wing” female, and the second mating between them. As another index of the preference for the polka-dotted pattern, we observed whether males courted females and which female was courted first. Also, courtship latencies (Latency 1 and 2) and courtship durations (Duration 1 and 2) were measured.

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

Preference for the polka-dotted pattern

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

Statistical analysis

All statistical analyses were performed using R ver. 3.5.2 (R Core Team, 2018). In both male and female mate choice experiments, GLMs were used to investigate the influence of the mating combination between the sexes, male age, female age and hours after females had received replaced wings (if the experiment included wing replacement) on the occurrence of mating. We included ages and hours after wing replacements in this analysis, because ages and CO_2 anaesthesia are known to affect the behavior of some *Drosophila* species (Avent, Price & Wedell, 2008; Colinet & Renault, 2012; Verspoor, Cuss & Price, 2015). The occurrence of mating was treated as a response variable assuming a binomial distribution. Mating combination, male age, female age and hours after females had received replaced wings were treated as explanatory variables. As a link function, logit function was used. In addition to GLMs, chi-squared tests were performed on the mating combination between the sexes without variables of ages. For the three experiments to test the preference for the polka-dotted pattern, Bonferroni correction with chi-squared test was performed to counteract the problem of multiple similar experiments (Wright, 1992). When sample size was less than 40, Yates correction with the chi-squared test was performed (Campbell, 2007). For comparison of courtship latencies and courtship durations, we used the Student's *t*-test. To determine which type of female was courted first and which type of male courted first, we used the exact binomial test.

Results

The courtship behaviour of *D. guttifera*

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

Sexual immaturity at 24 hours after eclosion

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

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

Preference for wings

For male choice experiments, we conducted 51 experiments to examine preference for wings (Figure 2a). Some males mated with both of the two females. Such matings were recorded separately as the first mating and the second mating. Males never mated twice with the same female in our observations. In the first matings, males mated with "wild type" females 25 times and with "no wing" females 10 times. In the second matings, males mated with "wild type" females four times and with "no wing" females 11 times. Thus, at the first mating, males mated with "wild type" at higher rate than with "no wing", and at the second mating males mated with "no wing" females at higher rate than with "wild type" females (GLM, estimate=-2.7865, *p*=0.000682; chi-squared test, *p*=0.0006539). Other explanatory variables were not statistically significant (GLM, *p*>0.05).

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

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

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

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

Preference for conspecific wings

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

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

For female choice experiments, we conducted 49 experiments to examine the preference for the polka-dotted *D. guttifera* wings (Figure 3b). Females mated with “*guttifera* wing” males 22 times and “*melanogaster* wing” males 12 times. Females mated with “*guttifera* wing” males significantly more frequently than with “*melanogaster* wing” males (GLM, estimate=0.94335, *p*=0.0361; chi-squared test, *p*=0.03382). Other explanatory variables were not statistically significant (GLM, *p*>0.05).

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

Preference for polka-dotted pattern

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

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

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

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

The courtship and mating rate in *D. guttifera*

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

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

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

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

Discussion

Preference for wings

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

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

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

Preference for conspecific wings

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

Preference for the polka-dotted pattern

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

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

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

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Conflict of interest

The authors declare that they have no conflict of interest.

References

Andersson, M. (1982). Female choice selects for extreme tail length in a widowbird. *Nature*, *299*(5886), 818-820. https://doi.org/10.1038/299818a0

Avent, T. D., Price, T. A. R., & Wedell, N. (2008). Age-based female preference in the fruit fly *Drosophila pseudoobscura*. *Animal Behaviour*, *75*(4), 1413-1421. https://doi.org/10.1016/j.anbehav.2007.09.015

Bell, A. M., Hankison, S. J., & Laskowski, K. L. (2009). The repeatability of behaviour: a meta-analysis. *Animal Behaviour*, *77*(4), 771-783. https://doi.org/10.1016/j.anbehav.2008.12.022

Bonduriansky, R. (2001). The evolution of male mate choice in insects: a synthesis of ideas and evidence. *Biological Reviews*, *76*(3), 305-339. https://doi.org/10.1017/s1464793101005693

Bonduriansky, R., & Chenoweth, S. F. (2009). Intralocus sexual conflict. *Trends in Ecology & Evolution*, *24*(5), 280-288. https://doi.org/10.1016/j.tree.2008.12.005

Byrne, P. G., & Rice, W. R. (2006). Evidence for adaptive male mate choice in the fruit fly *Drosophila melanogaster*. *Proceedings of the Royal Society B: Biological Sciences*, *273*(1589), 917-922. https://doi.org/10.1098/rspb.2005.3372

Campbell, I. (2007). Chi‐squared and Fisher–Irwin tests of two‐by‐two tables with small sample recommendations. *Statistics in Medicine*, *26*(19), 3661-3675. https://doi.org/10.1002/sim.2832

Cartar, R. V. (1992). Morphological senescence and longevity: an experiment relating wing wear and life span in foraging wild bumble bees. *Journal of Animal Ecology*, *61*(1), 225-231. https://doi.org/10.2307/5525

Chialvo, C. H. S., White, B. E., Reed, L. K., & Dyer, K. A. (2019). A phylogenetic examination of host use evolution in the *quinaria* and *testacea* groups of *Drosophila*. *Molecular Phylogenetics and Evolution*, *130*, 233-243. https://doi.org/10.1016/j.ympev.2018.10.027

Colinet, H., & Renault, D. (2012). Metabolic effects of CO2 anaesthesia in *Drosophila melanogaster*. *Biology Letters*, *8*(6), 1050-1054. https://doi.org/10.1098/rsbl.2012.0601

Combes, S. A., Crall, J. D., & Mukherjee, S. (2010). Dynamics of animal movement in an ecological context: dragonfly wing damage reduces flight performance and predation success. *Biology Letters*, *6*(3), 426-429. https://doi.org/10.1098/rsbl.2009.0915

Dion, W. A., Shittu, M. O., Steenwinkel, T. E., Raja, K. K., Kokate, P. P., & Werner, T. (2020). The modular expression patterns of three pigmentation genes prefigure unique abdominal morphologies seen among three *Drosophila* species. *Gene Expression Patterns*, 119132. https://doi.org/10.1016/j.gep.2020.119132

Dufour, H. D., Koshikawa, S., & Finet, C. (2020) Temporal flexibility of gene regulatory network underlies a novel wing pattern in flies. *Proceedings of the National Academy of Sciences of the United States of America*, *117*(21), 11589-11596. https://doi.org/10.1073/pnas.2002092117

Edwards, K. A., Doescher, L. T., Kaneshiro, K. Y., & Yamamoto, D. (2007). A database of wing diversity in the Hawaiian *Drosophila*. *PLoS One*, *2*(5), e487. https://doi.org/10.1371/journal.pone.0000487

Fisher, R. A. (1915). The evolution of sexual preference. *The Eugenics Review*, *7*(3), 184-192.

Fukutomi, Y., & Koshikawa, S. (2021). Mechanism of color pattern formation in insects. In H. Hashimoto, M. Goda, R. Futahashi, R. Kelsh & T. Akiyama (Eds.), *Pigments, pigment cells, pigment patterns*. Singapore: Springer. (in press)

Fukutomi, Y., Kondo, S., Toyoda, A., Shigenobu, S., & Koshikawa, S. (2021). Transcriptome analysis reveals *wingless* regulates neural development and signaling genes in the region of wing pigmentation of a polka-dotted fruit fly. *FEBS Journal*, *288*(1), 99-110. https://doi.org/10.1111/febs.15338

Fukutomi, Y., Matsumoto, K., Agata, K., Funayama, N., & Koshikawa, S. (2017). Pupal development and pigmentation process of a polka-dotted fruit fly, *Drosophila guttifera* (Insecta, Diptera). *Development Genes and Evolution*, *227*(3), 171-180. https://doi.org/10.1007/s00427-017-0578-3

Fukutomi, Y., Matsumoto, K., Funayama, N., & Koshikawa, S. (2018). Methods for staging pupal periods and measurement of wing pigmentation of *Drosophila guttifera*. *JoVE (Journal of Visualized Experiments)*, (131), e56935. https://doi.org/10.3791/56935

Fuyama, Y. (1979). A visual stimulus in the courtship of *Drosophila suzukii*. *Experientia*, *35*(10), 1327-1328. https://doi.org/10.1007/BF01963987

Grossfield, J. (1966). The influence of light on the mating behaviour of *Drosophila*. In M. R. Wheeler (Ed.), Studies in Genetics III (pp. 147-176). Austin, Texas: The University of Texas Publication.

Grossfield, J. (1977). *Drosophila* courtship: decapitated quinaria group females. *Journal of the New York Entomological Society*, *85*(3), 119-126.

Hegde, S. N., Chethan, B. K., & Krishna, M. S. (2005). Mating success of males with and without wing patch in *Drosophila biarmipes*. *Indian Journal of Experimental Biology*, *43*(10), 902-909.

Izumitani, H. F., Kusaka, Y., Koshikawa, S., Toda, M. J., & Katoh, T. (2016). Phylogeography of the subgenus *Drosophila* (Diptera: Drosophilidae): evolutionary history of faunal divergence between the Old and the New Worlds. *PLoS One*, *11*(7), e0160051. https://doi.org/10.1371/journal.pone.0160051

Jantzen, B., & Eisner, T. (2008). Hindwings are unnecessary for flight but essential for execution of normal evasive flight in Lepidoptera. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(43), 16636-16640. https://doi.org/10.1073/pnas.0807223105

Kopp, A., & True, J. R. (2002). Evolution of male sexual characters in the oriental *Drosophila melanogaster* species group. *Evolution & Development*, *4*(4), 278-291. https://doi.org/10.1046/j.1525-142x.2002.02017.x

Koshikawa, S. (2020). Evolution of wing pigmentation in *Drosophila*: Diversity, physiological regulation, and *cis*-regulatory evolution. *Development, Growth & Differentiation*, *62*(5), 269-278. https://doi.org/10.1111/dgd.12661

Koshikawa, S., Fukutomi, Y., & Matsumoto, K. (2017). *Drosophila guttifera* as a model system for unraveling color pattern formation. In T. Sekimura & H. F. Nijhout (Eds.), *Diversity and Evolution of Butterfly Wing Patterns* (pp. 287-301). Singapore: Springer.

Koshikawa, S., Giorgianni, M. W., Vaccaro, K., Kassner, V. A., Yoder, J. H., Werner, T., & Carroll, S. B. (2015). Gain of cis-regulatory activities underlies novel domains of wingless gene expression in *Drosophila*. *Proceedings of the National Academy of Sciences of the United States of America*, *112*(24), 7524-7529. https://doi.org/10.1073/pnas.1509022112

Kraaijeveld, K., Kraaijeveld-Smit, F. J., & Komdeur, J. (2007). The evolution of mutual ornamentation. *Animal Behaviour*, *74*(4), 657-677. https://doi.org/10.1016/j.anbehav.2006.12.027

Lande, R. (1980). Sexual dimorphism, sexual selection, and adaptation in polygenic characters. *Evolution*, *34*(2), 292-305. https://doi.org/10.1111/j.1558-5646.1980.tb04817.x

Miller, M. E., Marshall, S. A., & Grimaldi, D. A. (2017) A review of the species of *Drosophila* (Diptera: Drosophilidae) and genera of Drosophilidae of Northeastern North America. *Canadian Journal of Arthropod Identification*, 31. https://doi.org/10.3752/cjai.2017.31

Nolan, P. M., Dobson, F. S., Nicolaus, M., Karels, T. J., McGraw, K. J., & Jouventin, P. (2010). Mutual mate choice for colorful traits in king penguins. *Ethology*, *116*(7), 635-644. https://doi.org/10.1111/j.1439-0310.2010.01775.x

Parkash, R., Lambhod, C., & Singh, D. (2013). Ecological significance of wing spot dimorphism in *Drosophila biarmipes* (Diptera, Drosophilidae). *Acta Entomologica Sinica*, *56*(11), 1267-1274.

Patterson, J. T. (1943). The Drosophilidae of the Southwest. Austin, Texas: The University of Texas Publication.

Petrie, M., & Halliday, T. (1994). Experimental and natural changes in the peacock's (*Pavo cristatus*) train can affect mating success. *Behavioral Ecology and Sociobiology*, *35*(3), 213-217. https://doi.org/10.1007/BF00167962

Petrie, M., Halliday, T., & Sanders, C. (1991). Peahens prefer peacocks with elaborate trains. *Animal Behaviour*, *41*(2), 323-331. https://doi.org/10.1016/S0003-3472(05)80484-1

Pincemy, G., Dobson, F. S., & Jouventin, P. (2009). Experiments on colour ornaments and mate choice in king penguins. *Animal Behaviour*, *78*(5), 1247-1253. https://doi.org/10.1016/j.anbehav.2009.07.041

Prud'Homme, B., Gompel, N., Rokas, A., Kassner, V. A., Williams, T. M., Yeh, S. D., True, J. R., & Carroll, S. B. (2006). Repeated morphological evolution through cis-regulatory changes in a pleiotropic gene. *Nature*, *440*(7087), 1050-1053. https://doi.org/10.1038/nature04597

R Core Team (2018). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. Retrieved from https://www.R-project.org/

Roy, P. R., & Gleason, J. M. (2019). Assessing the use of wing ornamentation and visual display in female choice sexual selection. *Behavioural Processes*, *158*, 89-96. https://doi.org/10.1016/j.beproc.2018.10.010

Ryan, M. J. (1985). The túngara frog: a study in sexual selection and communication. Chicago: University of Chicago Press.

Shittu, M., Steenwinkel, T., Koshikawa, S., Werner, T. (2020). The making of transgenic *Drosophila guttifera*. *Methods and Protocols*, 3(2), 31. https://doi.org/110.3390/mps3020031

Singh, B. N., & Chatterjee, S. (1987). Greater mating success of *Drosophila biarmipes* males possessing an apical dark black wing patch. *Ethology*, *75*(1), 81-83. https://doi.org/10.1111/j.1439-0310.1987.tb00643.x

Spieth, H. T. (1952). Mating behaviour with in genus *Drosophila* (Diptera). New York: Bulletin of American Museum of Natural History.

Spieth, H. T. (1966). Courtship behavior of endemic Hawaiian *Drosophila*. Austin, Texas: The University of Texas Publication.

Theis, A., Salzburger, W., & Egger, B. (2012). The function of anal fin egg-spots in the cichlid fish *Astatotilapia burtoni*. *PLoS One*, *7*(1), e29878. https://doi.org/10.1371/journal.pone.0029878

Verspoor, R. L., Cuss, M., & Price, T. A. R. (2015). Age-based mate choice in the monandrous fruit fly *Drosophila subobscura*. *Animal Behaviour, 102,* 199-207. https://doi.org/10.1016/j.anbehav.2015.01.024

Wen, S. Y., & Li, Y. F. (2011). An evolutionary view on courtship behavior of *Drosophila*: from a comparative approach. *Low Temperature Science*, *69,* 87-100.

Werner, T., Koshikawa, S., Williams, T. M., & Carroll, S. B. (2010). Generation of a novel wing colour pattern by the Wingless morphogen. *Nature*, *464*(7292), 1143-1148. https://doi.org/10.1038/nature08896

Werner, T., Steenwinkel, T., & Jaenike, J. (2020). The encyclopedia of North American Drosophilids Volume 1: Drosophilids of the Midwest and Northeast. Houghton, MI: Michigan Technological University.

Wright, S. (1992). Adjusted p-values for simultaneous inference. *Biometrics, 48*(4), 1005-1013. https://doi.org/10.2307/2532694

Zahavi, A. (1975). Mate selection—a selection for a handicap. *Journal of Theoretical Biology*, *53*(1), 205-214. https://doi.org/10.1016/0022-5193(75)90111-3

Figure legends

Figure 1.

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

Figure 2.

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

Figure 3.

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

Figure 4.

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

Table 1.

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

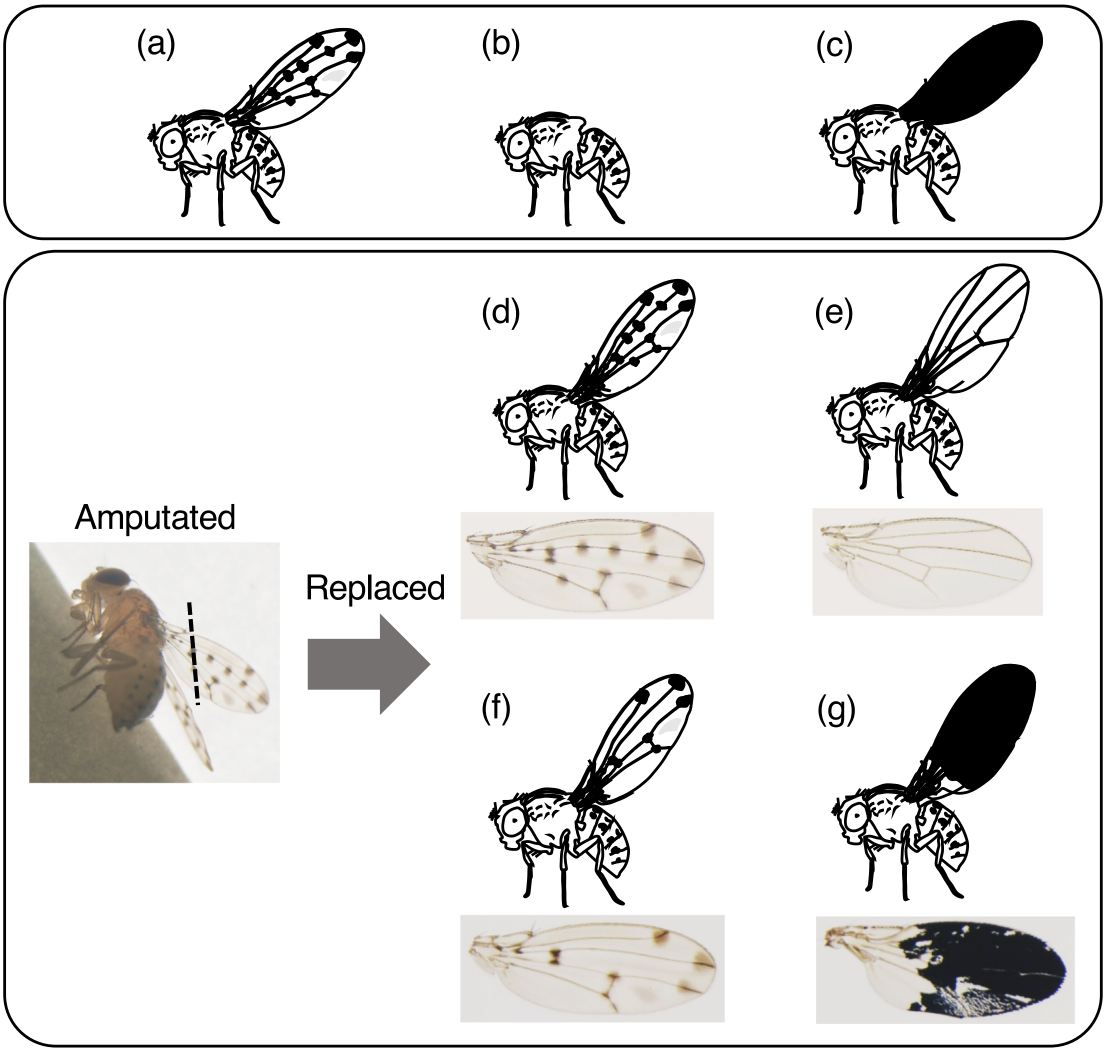

Figure 1

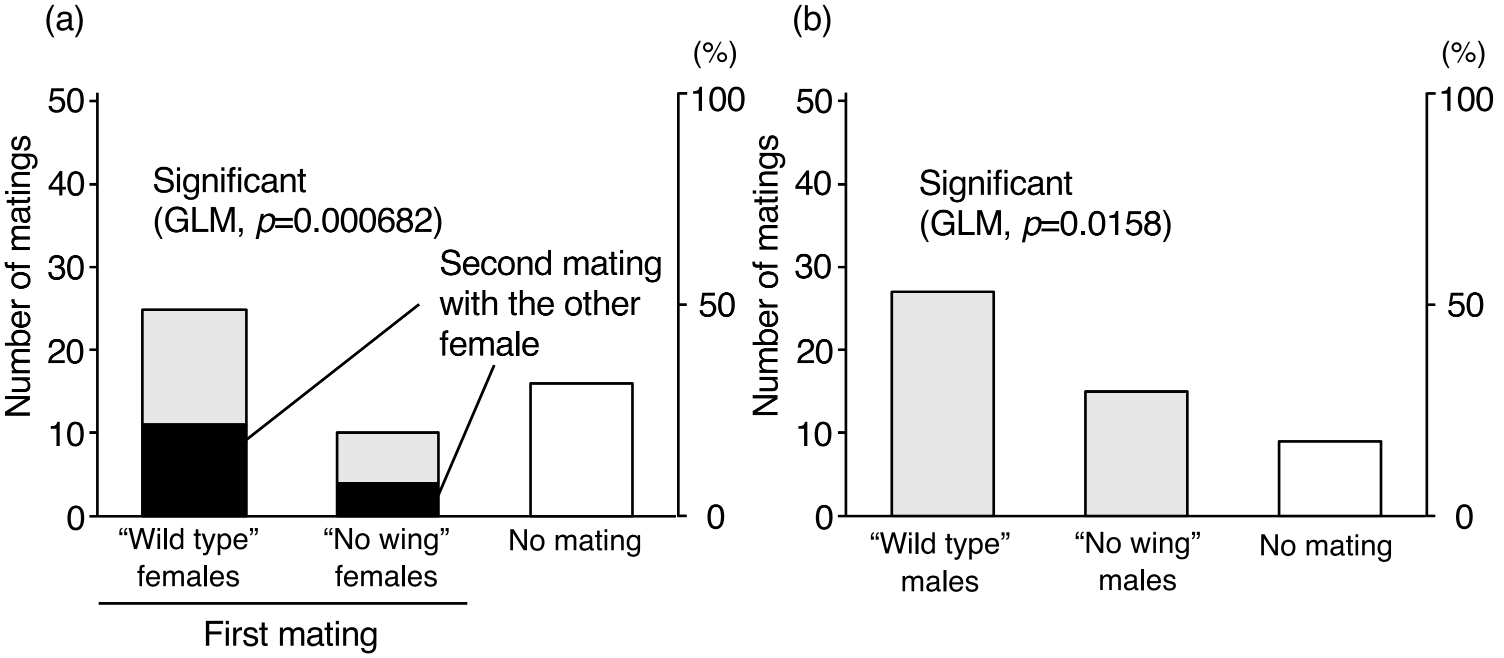
(a)
(%)
Number of matings
0
10
20
30
40
50
0
50
100
Significant
(GLM, *p*=0.000682)
Second mating
with the other
female
"Wild type"
females
"No wing"
females
No mating
First mating
(b)
(%)
Number of matings
0
10
20
30
40
50
0
50
100
Significant
(GLM, *p*=0.0158)
"Wild type"
males
"No wing"
males
No mating

Figure 2

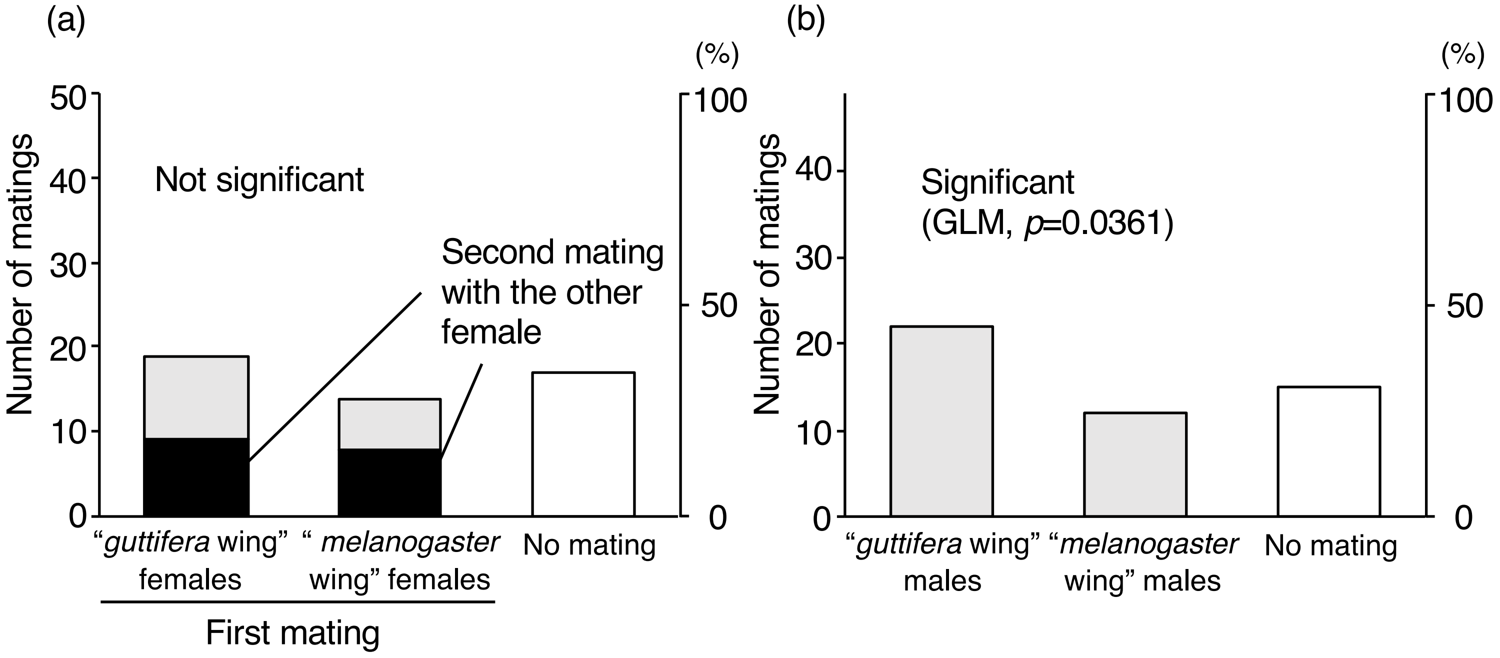
(a)
(%)
50
40
30
20
10
0
100
50
0
Number of matings
Not significant
Second mating with the other female
"*guttifera* wing" females
"*melanogaster* wing" females
No mating
First mating
(b)
(%)
40
30
20
10
0
100
50
0
Number of matings
Significant (GLM, *p*=0.0361)
"*guttifera* wing" males
"*melanogaster* wing" males
No mating

Figure 3

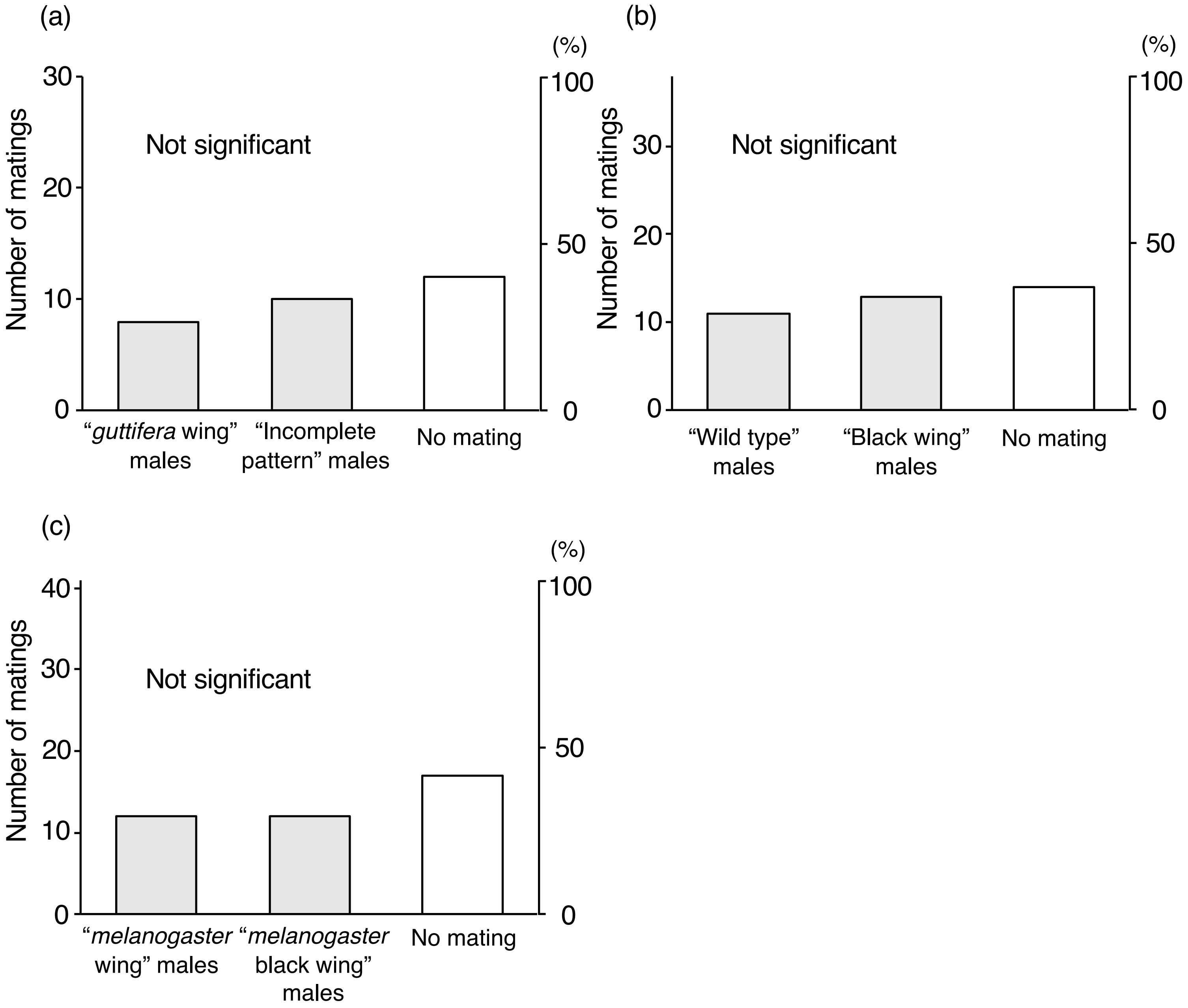
(a)
(%)
Number of matings
30
20
10
0
100
50
0
Not significant
"*guttifera* wing" males
"Incomplete pattern" males
No mating
(b)
(%)
Number of matings
30
20
10
0
100
50
0
Not significant
"Wild type" males
"Black wing" males
No mating
(c)
(%)
Number of matings
40
30
20
10
0
100
50
0
Not significant
"*melanogaster* wing" males
"*melanogaster* black wing" males
No mating

Figure 4

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

Table 1