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Fruit fly courtship: The female perspective

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Abstract Research on fruit fly courtship has mostly focused on males' behavioral repertoire whereas females have been assumed to respond by either rejecting or accepting males. In many fruit fly species including *Drosophila melanogaster*, however, mating typically follows an extended period of courtship, which provides ample opportunities for females to inform males about their likelihood of mating. Our experiments indeed revealed that sexually immature females in both *D. melanogaster*and *D. simulans* showed responses to conspecific males that were distinct from those of sexually mature females. Furthermore, females' responses to conspecific males were different from their responses to heterospecific males. Our data indicate that females' behavioral repertoire early in courtship can inform males about their probability of mating if they persist in courting. We hypothesize (i) that males can rely on behavioral feedback from females for optimally allocating their courtship efforts towards distinct female classes, (ii) that males may learn to modulate their courtship behavior based on specific feedback from females, and (iii) that females may learn to alter their behavior towards distinct types of males in order to elicit the desired male response. Overall, we suggest that, although little explored, female behavior determines the dynamics of courtship and mating and can thus influence sexual selection and incipient speciation [*Current Zoology* 61 (6): 1008–1014, 2015].

Keywords Courtship, Female behavior, Fruit flies, Learning, Mate choice, Speciation

The dynamics of pre-mating interactions between males and females influence the strength and direction of sexual selection as well as mechanisms of reproductive isolation that lead to speciation (Andersson, 1994; Coyne and Orr, 2004). While there is tremendous variation among species in those dynamics, the most recognized pattern involves male courtship culminating with either mating or rejection by the female. For example, numerous studies in fruit flies (*Drosophila* spp) have focused on minute details of male courtship behaviour and their outcomes: either acceptance or rejection by females (Hall, 1994; Dickson, 2008). Although the females' behavioral repertoire has been well described (Spieth, 1952), we know relatively little about the information content of such behaviors. Notable exceptions are the well-defined ovipositor extrusion by recently mated females, which repels courting males (Spieth, 1952; Bastock and Manning, 1955; Connolly and Cook, 1973), and the behavior preceding mating, which includes reduced movement and opening of the vaginal plates (Spieth, 1952; Markow and Hanson, 1981; Lasbleiz et al., 2006). We should note, however, that almost all studies on male courtship and female response were carried out with flies confined to tiny test chambers, which most likely limited male and female behavioural repertoires and their dynamics.

In many species of fruit flies including the best studied, *D. melanogaster*, females typically accept males as mates only after an extended period of courtship display. The females, however, provide immediate feedback to the males and continue to do so during courtship. Such female responses can inform the males about their likelihood of mating if they persist in courting and thus affect their subsequent courtship effort and strategy. Because persistent courtship is typically a pre-requisite for acceptance by females, its duration and quality can determine the frequency of mating between fly types. For example, if specific feedback from a female category informs males about a low probability of mating, they may learn to reduce courtship effort towards such females and hence reduce the frequency of inter-type matings (Dukas, 2008; Kujtan and Dukas, 2009).

Perhaps the best studied species in which female feedback has been shown to influence male courtship is the brood parasite, brown headed cowbird *Molothrus ater*. During courtship by young males, certain song types elicit the female to respond with a wing stroke, a rapid lateral movement of the wing away from the body, which resembles the initial wing movement leading to copulatory posture. Males are more likely to subse-

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quently sing the song type eliciting the wing stroke (West and King 1988), and such feedback can shape male song repertoire (King and West, 1983). Similarly, in satin bowerbirds *Ptilonorhynchus violaceus*, more intense male displays increase mating success as long as they do not startle females. Males that modulated their courtship intensity based on female response were less likely to startle the female and had a higher mating success (Patricelli et al., 2002).

The data just reviewed from two thoroughly examined avian model systems suggest that female behaviours during courtship as well as male responses to such behaviours can determine both male mating success and the frequency of matings between distinct categories of individuals. Hence quantifying the information content of female behavior during courtship can help us understand processes influencing sexual selection and incipient speciation. To address this issue, we conducted experiments with fruit flies (*D. melanogaster* and *D. simulans*) to answer two basic questions. First, does female behaviour cue males about their likelihood of mating with either a given individual female or that female category? Second, do males rely on cues from females for determining their courtship decisions? A unique feature of our protocol was the use of relatively large cages, which enabled free movement of flies. This allowed us to quantify female decamping, a prominent behavior observed in the field, as well as male termination of courtship, which, as far as we know, has not been measured previously.

1 Materials and Methods

1.1 General

We used three fly populations. The *D. melanogaster* flies were either descendants of flies caught at multiple sites in Southern Ontario half a year prior to the experiment, or Canton-S flies, which have been in the lab for decades. The *D. simulans* flies were descendants of flies collected in Southern California in 2009. We kept flies in large population cages each containing several hundred flies supplied with media containing sucrose, yeast, cornmeal, water, agar and methyl paraben under a 12:12 light dark cycle at 25°C and 60% RH (Dukas and Dukas, 2012). While the *Canton-S* and *D. simulans* flies had been kept in vials prior to arrival in our laboratory, they spent 5 years and 6 months respectively in our population cages. We used sexually mature, 3 day old males. Based on preliminary trials with the three fly lines used in our experiments, we used immature females that were younger than 19 h and hence unreceptive, and young, sexually mature females that were 24–38 h old. We used flies that had not encountered flies of the other sex prior to the test and used each individual only once.

Field observations by us and others (e.g. Spieth, 1974; Spieth and Ringo, 1983) indicated that courtship in natural settings is distinct from that observed in the confined space of small cells. We thus elected to use cubic Plexiglas cages with 10 cm walls, with one wall made of a screen sleeve, which allowed easy access into the cage. On the floor next to the wall opposite the screen, we placed a cylinder of food medium 22 mm in diameter and 5 mm tall covered with live yeast suspension. An LED light shone on the food to enhance fly attraction to that spot.

We conducted all observations between 8 AM to noon, which corresponded to early morning under our photoperiod. We ran all treatments at the same time at a random, counterbalanced order. We began each trial by aspirating a focal male and four females of the same age and species. Our protocol thus simulated a realistic setting in whic h males may encounter several females in their vicinity, and females can readily express their full range of behavioral repertoire, including the termination of males' advances by decamping. Using a custom-made computer program, observers blind to fly type recorded male and female behaviour for 5 min starting with the first courtship, which usually occurred within a few minutes. We recorded all starts and ends of male courtship bouts and later calculated the proportion of time males spent courting as well as their rate of aborting courtship. We considered courtship abortion any case where it was the male rather than the female who terminated courtship, for example, by walking away from the female or by ceasing to follow a walking female.

Females either appeared to continue with their pre-courtship activity, most often feeding, or clearly responded to males. We recorded three distinct female rejection behaviors. Wing fluttering (Spieth, 1952), a clear vibration of the two wings, has also been named flicking (Bastock and Manning, 1955). Abdomen raising involves upwards movement of the tip of the abdomen (called twisting by Bastock and Manning, 1955). Finally, decamping means jumping or flying away (Spieth, 1952). While we also recorded whether females were feeding, walking, or resting on the cage wall, we had decided a priori to focus on the three explicit female rejection behaviours. Note that we did not record extruding (Spieth, 1952; Bastock and Manning, 1955; Connolly and Cook, 1973), the well documented rejection response of recently mated females, because we tested only virgin females. We calculated for each female the proportion of time spent wing fluttering and abdomen raising, and the decamping rate (number per minute). We calculated the proportions of times and rates based on the total time available for courtship, which was 5 min in trials with no matings and the mating latency in trials with matings. We analyzed the data with a generalized linear model with gamma distribution and log link function using IBM SPSS (IBM-Corp. 2011).

1.2 Behaviour of immature and mature *D. melanogaster* females towards conspecific males and the males' response

We began by comparing the behaviour of sexually immature and sexually mature females towards males, as well as the males' responses. We used the descendants of recently caught wild *D. melanogaster* in this experiment and conducted 71 trials. There were no matings with immature females while half of the mature females mated during the trials.

1.3 Behaviour of immature and mature *D. simulans* females towards conspecific and heterospecific males and the males' response

Often, male fruit flies searching for prospective mates encounter a mixture of potentially receptive conspecific and unreceptive heterospecific females. If the heterospecific females employ distinct behaviors from those of conspecific females, they can lead the males to quickly terminate heterospecific courtship. Hence to broaden our investigation, we tested for differential responses of mature and immature virgin females towards conspecific males in another species, *D. simulans*, and also tested these females' responses to males of the closely related *D. melanogaster*. In this experiment, the *D. melanogaster* males belonged to the Canton-S population. We used only a single heterospecific species pairing because, in our lines, male *D. melanogaster* readily court female *D. simulans*, who reject them, but male *D. simulans* rarely court female *D. melanogaster*. We conducted 80 trials in which we recorded neither heterospecific matings nor matings with immature females and 30% conspecific matings with mature females.

2 Results

2.1 Behaviour of immature and mature *D. melanogaster* females towards conspecific males and the males' response

There was a major difference between immature and mature females, with the former showing twice as much wing fluttering (Wald $\chi^2_1 = 5.15$, $n = 71$, $P < 0.05$) and the latter having twice the decamping rates (Wald $\chi^2_1 = 5.09$, $n = 71$, $P < 0.05$, Fig. 1). We recorded only a few cases of abdomen raising. The males spent similar proportions of time courting immature and mature females (0.6 ± 0.047 and 0.7 ± 0.049 respectively; Wald $\chi^2_1 = 0$, $n = 71$, $P = 1$) and were equally likely to abort courting immature and mature females (0.4 ± 0.06 and 0.5 ± 0.1 respectively; Wald $\chi^2_1 = 0.9$, $n = 71$, $P = 0.3$).

2.2 Behaviour of immature and mature female *D. simulans* towards conspecific and heterospecific males

Overall, females gave more rejection signals to heterospecific than to conspecific males. There was also a major difference between immature and mature females, with the former showing more wing fluttering and the latter having higher decamping rates. Specifically, first, females spent more time raising their abdomen in response to heterospecific than to conspecific males (Wald $\chi^2_1 = 14.6$, $n = 80$, $P < 0.001$, Fig. 2A). Female age (immature vs mature) had no effect on the time spent abdomen raising (Wald $\chi^2_1 = 1$, $n = 80$, $P = 0.3$). Second, females spent more time wing fluttering in response to heterospecific than to conspecific males (Wald $\chi^2_1 = 11.3$, $n = 80$, $P < 0.001$) and immature females fluttered more than mature females (Wald $\chi^2_1 = 18.8$, $n = 80$, $P < 0.001$, Fig. 2B). Finally, females decamped more often when courted by heterospecific than by conspecific males (Wald $\chi^2_1 = 22.3$, $n = 80$, $P < 0.005$, Fig. 2C). Regardless of the male species courting them (conspecific *vs* heterospecific), immature females decamped at a lower rate than mature females (Wald $\chi^2_1 = 4.4$, $n = 80$,

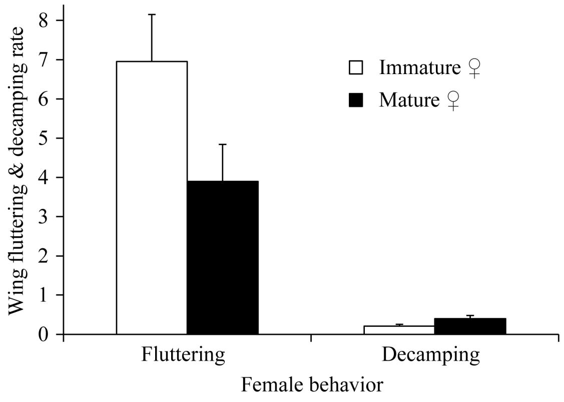

Fig. 1 The proportion of time spent wing fluttering and rate of decamping by immature and mature female *D. melanogaster* in response to courting conspecific males

The bars depict mean ± 1 *SE*. *n* = 71 trials each involving one male and four females of the same category (either four immature or four mature females).

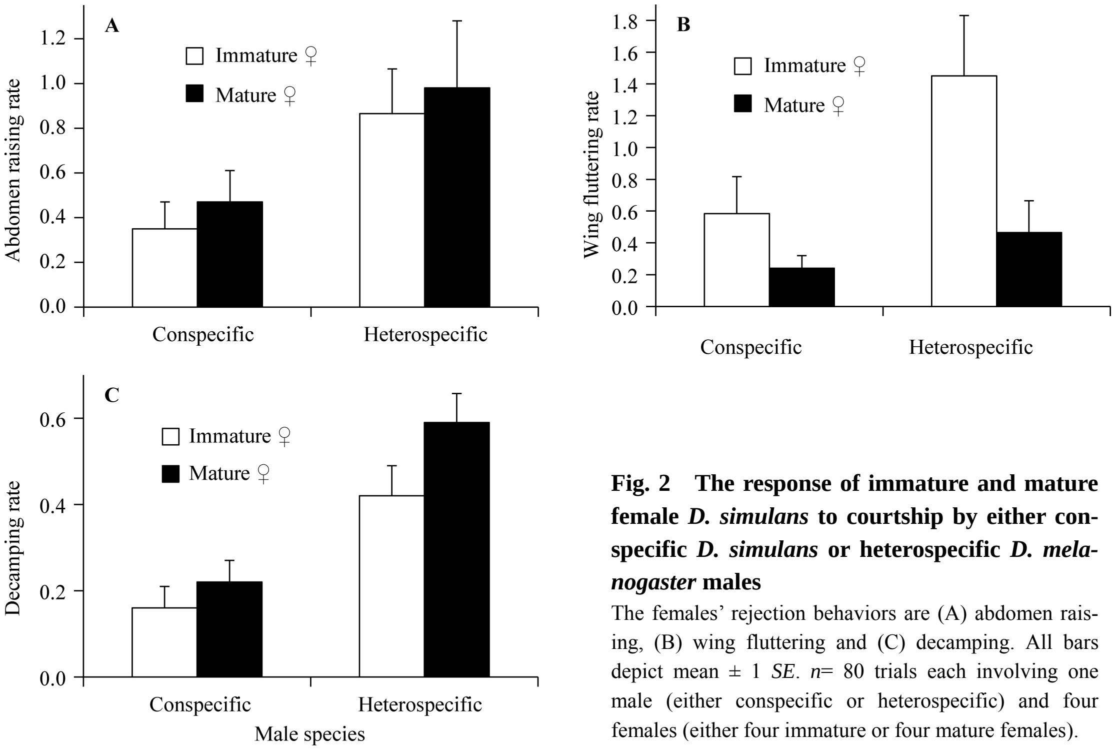

Fig. 2 The response of immature and mature female *D. simulans* to courtship by either conspecific *D. simulans* or heterospecific *D. melanogaster* males

The females' rejection behaviors are (A) abdomen raising, (B) wing fluttering and (C) decamping. All bars depict mean ± 1 *SE*. n= 80 trials each involving one male (either conspecific or heterospecific) and four females (either four immature or four mature females).

$P < 0.05$ for female effect, Wald $\chi^2_1 = 0.09$, $n = 80$, $P = 0.8$ for male by female interaction, Fig. 2C).

The conspecific males spent a higher proportion of time courting mature females than did the heterospecific males but the heterospecific males spent twice as much time courting immature females than did the conspecific males (Wald $\chi^2_2 = 10.6$, $n = 80$, $P < 0.001$ for the male by female interaction, Fig. 3A). The frequency at which males stopped pursuing females showed a parallel male by females interaction, with conspecific males quitting courtship less often with mature than with immature females and heterospecific males quitting courtship less often with immature than with mature females (Wald $\chi^2_2 = 3$, $n = 80$, $P = 0.08$ for the male by female interaction, Fig. 3B).

3 Discussion

3.1 Female behavior

Our results indicate that males initiating courtship receive immediate behavioral feedback that indicate the receptivity of their prospectivemates. In both *D. melanogaster* and *D. simulans*, immature females showed responses distinct from those of mature virgin females, and *D. simulans* females responded differently to courtship from conspecific and heterospecific males (Figs 1, 2). While the distinct response of immature and mature virgin females could merely indicate age-specific variation in behaviour, the fact that females responded differ-

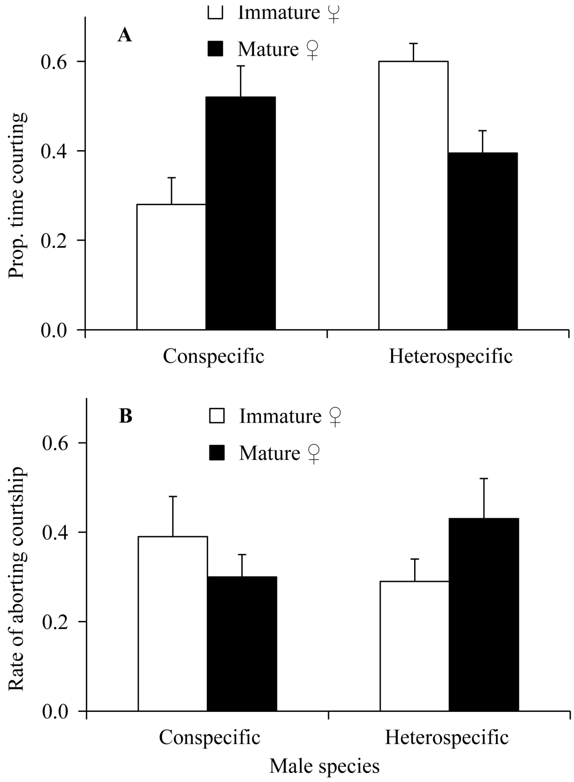

Fig. 3 The proportion of time (A) either conspecific *D. simulans* or heterospecific *D. melanogaster* males spent courting either immature or mature female *D. simulans* and the rate at which the males terminated courtship bouts (B)

All bars depict mean ± 1 *SE*. n = 80 trials each involving one male (either conspecific or heterospecific) and four females (either four immature or four mature *D. simulans* females).

rently to conspecific and heterospecific males (Fig. 2) provides concrete evidence that females modulate their suite of behaviors based on the specific characteristics of the males that court them. Such male characteristics may include odor, song and behaviour. Female behavior has been well described previously (Spieth, 1952) and at least one study reported more wing fluttering in immature than in mature females *D. melanogaster* (Connolly and Cook, 1973). We know, however, of no other study that examined distinct female behavioral responses to conspecific versus heterospecific males.

Based on field observations, we ruled out the use of small observation chambers for our study. Such small arenas severely limit fly movement and have probably biased previous studies on male-female interactions during courtship. The cost of conducting observations in relatively large cages, however, was our inability to have high-quality videos for a refined analysis of behavior. It is thus possible that we have missed subtle features of female responses to males. For example, it is possible that, rather than being an overt signal of rejection, decamping is actually a continuous trait, in which short-distance hops are part of the courtship dynamics whereas long flights serve to evade males.

Another limitation of our protocol was that it could not accommodate the delicate hardware necessary for sound recording. Hence, like our predecessors, we visually recorded females' wing fluttering even though audio-recording might reveal context-specific variation in sound cues. While the auditory signals produced by male fruit flies during courtship have been extensively studied (Ewing and Bennet-Clark, 1968; Von Schilcher, 1976; Coen et al., 2014), there has been very little research on female-generated sounds in *Drosophila* (Donegan and Ewing, 1980; Satokangas et al., 1994) and we know of only a single study that briefly mentions female sounds in *D. melanogaster* (Ewing and Bennet-Clark, 1968). Interestingly, Ewing and Bennet-Clark (1968) noted that the female sound they recorded was produced with "wings folded and often without observable movement of the wings". This further emphasizes the need for a follow-up acoustic analysis focusing on the role of female sounds during courtship.

Finally, our protocol and observations followed the convention of assuming that males pursue females and females respond to males. We know, however, that this is not the case in natural settings with relatively low fly density, where capable males defend decaying fruit that attract females (Markow, 1988). In such a classical mating-system of resource-defence polygyny (Emlen and Oring, 1977; Baxter et al., 2015), the role of female behaviour may be different than in the typical mating system under high fly density, which involves scramble competition among males for females (Spieth, 1974; Thornhill and Alcock, 1983). Although we do not know the relative prevalence of resource-defence polygyny in *D. melanogaster*, the very strong tendency of males of this as well as other *Drosophila* spp. to defend resources where feasible (Hoffmann, 1987; Markow, 1988) suggests that its contribution to sexual selection and incipient speciation is substantial. Hence the role of female behavior in the context of a resource defence mating system should receive close attention.

Gowaty and colleagues (Gowaty et al., 2002, 2003) adopted an original approach to male-female interactions in three species of fruit flies including *D. melanogaster*. Their primary behavioral measure was "interest" in the other fly, defined as the percentage of an individual's total changes of direction that were toward the other fly inside a 40 ml food vial. Unfortunately, it is not clear whether a vial-mate was within the short distance necessary for perceiving the other fly, and whether this unique measure reflects a fly's sexual interest in its vial-mate. Nevertheless, we concur with the main message of the studies by Gowaty and colleagues (Gowaty et al., 2002, 2003), which is that female fruit flies have more active role in courtship than has been portrayed in the literature.

3.2 Male response

Our data indicate that female response to courting males can inform the males about their likelihood of mating with the female they are courting. In the first experiment comparing male *D. melanogaster* courtship persistence with immature and mature virgin conspecifics, the males did not show the expected adaptive behavior of courting immature females less than mature virgin females. That is, in spite of the distinct behavioral (Fig. 1) and, most likely, different odor and gustatory cues from immature and mature females (Montell, 2009; Arienti et al., 2010; Everaerts et al., 2010), the males did not modulate their courtship effort. Male *D. simulans*, however, did show the expected lower courtship of immature than of mature virgin females (Fig. 3A, left pair of bars). While we cannot explain this species difference, our data are consistent with other studies, which indicate that male *D. melanogaster* show intense courtship of flies with ambiguous characteristics, including young males and females, which lack the characteristic cuticular hydrocarbons of mature flies (McRobert and Tompkins, 1983; Arienti et al., 2010; Dukas, 2010), and females that do not produce most of their cuticular hydrocarbons (Billeter et al., 2009; Dweck et al., 2015). The finding that male *D. melanogaster* spent more time courting immature than mature female *D. simulans* (Fig. 3A, right pair of bars) is also in agreement with our generalization that male *D. melanogaster* are highly attracted to sexually ambiguous flies.

3.3 Female behavior, learning, sexual selection and speciation

It is well established that fruit flies as well as many other species learn in the context of courtship and mate choice and that such learning can affect the strength and direction of sexual selection and behaviors contributing to reproductive isolation (Siegel and Hall, 1979; Dukas, 2005; Dukas, 2008; Verzijden et al., 2012; Servedio and Dukas, 2013). In fruit flies, most studies have assumed that males associate female stimuli such as cuticular hydrocarbons and cis vaccenyl acetate with the outcome of their courtship, either mating with or rejection by the female (Dukas and Dukas, 2012). Our results indicate that females also provide a multitude of behavioral cues, which indicate their sexual receptivity. It is likely that males can rely on such cues for learning in the context of sexual behavior. Unlike the somewhat passive cuticular hydrocarbons, female behavioral cues provide males with active, direct feedback about their probability of mating. Learning to respond to such cues can enhance male fitness under some environmental conditions (Dukas et al., 2006; Servedio and Dukas, 2013).

It is possible that, like in the cowbird and bowerbirds studies mentioned in the introduction (West and King, 1988; Patricelli et al., 2002), male fruit flies rely on feedback from females to modulate their courtship (Morier-Genoud and Kawecki, 2015). Recent data indeed indicate that the males' courtship song is more plastic than previously thought, and that males' pattern of alternation between sine and pulse songs is affected by female movement (Trott et al., 2012; Coen et al., 2014). It is thus possible that males can learn to increase the frequency of the courtship features for which they receive positive feedback from females and decrease the components that lead to negative responses. Similarly, females may learn to increase the frequency of behaviors that cause unwanted males to quit courtship and induce attractive males to intensify their pursuit. The possible effects of such learning on sexual selection and incipient speciation are an exciting topic for future research.

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