



Mating success in fruit flies: courtship interference versus female choice

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The outcomes of sexual selection often differ when mating success is determined by male contest rather than female choice. Many studies, however, inferred sexual selection driven by female choice without carefully assessing the role of subtle male aggression. Relying on close-up video analyses, we documented novel courtship interference between male fruit flies, a key model system in research on sexual selection, sexual conflict and speciation. In experiments comparing male mating success under choice (2 males + 1 female) and no-choice (1 male + 1 female) conditions, we found that, in some cases, courtship interference altered male mating success. Both choice and no-choice protocols have known weaknesses. Choice protocols do not control for male–male interactions while no-choice protocols do not allow females to compare and choose between males. To overcome these weaknesses, we developed a new protocol (true-choice), which allows females to freely visit and assess each of two males while preventing direct male–male interactions. Results from the true-choice protocol suggest that traits enhancing male aggression have a greater role in determining mating success in fruit flies. Furthermore, it is possible that the mating system of scramble competition observed in many species should be reclassified as subtle male contest, which can drive sexual selection for aggressive male features.

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In many animals, the evolution of sexually selected traits is driven solely by either contests among males for access to females or females' choices of mates among the males that they encounter. In such clear cases, one can safely focus on either intra- or inter-sexual selection. Often, however, sexual selection is determined by a combination of male–male contests and female choice (Andersson, 1994; Hunt, Breuker, Sadowski, & Moore, 2009; Qvarnström & Forsgren, 1998; Shuker & Simmons, 2014). If the same traits, such as body size or dominance, are favoured by both male–male contests and female choice, then the distinction between the two mechanisms may be negligible. If different features, however, determine which males have access to females and which males are preferred by females, then it is crucial that we assess the separate contributions to sexual selection of male–male interactions and female choice.

Quantifying the distinct effects of male contest and pure female choice is challenging, and attempts at doing so have had variable

success determined by species' natural histories and the experimental protocols employed. An example of a successful attempt involves the scarlet-tufted malachite sunbird, *Nectarinia johnstoni*, in which field observations supplemented by experiments indicate that the males' scarlet pectoral tufts are important in male contests over territories whereas the males' tail lengths affect female choice (Evans & Hatchwell, 1992a, 1992b). Often, attempts to separate female choice from male contest involve species in which interactions are assumed to be primarily visual or auditory. In the visual species, partitions between males and females and tests for female proximity to each male might indicate female choice while controlling for male contest (e.g. Bischoff, Gould, & Rubenstein, 1985; Zuk, Johnson, Thornhill, & Ligon, 1990). Similarly, use of speakers for testing species with acoustic courtship eliminates male contest (e.g. Eriksson & Wallin, 1986; Ryan, 1980). However, in many animals including the species that rely on visual or auditory features, physical contact is an integral component of courtship. Such contact allows for the exchange of olfactory, gustatory and somatosensory information (e.g. Ferveur, 2010; Hughes, Harrison, & Gallup, 2007; Johnston, 2003; Lack, 1940; Włodarski & Dunbar, 2014). In such species, it is challenging to provide females with choice while simultaneously eliminating male–male interactions.

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The most common method for addressing female choice in species in which contact is part of the courtship ritual is to simply assume negligible effects of male contest. This indeed has been the standard protocol in numerous experiments on fruit flies (*Drosophila melanogaster*), a key model system in research on mate choice, sexual conflict and speciation (Coyne & Orr, 2004; Rice et al., 2006). In such choice studies, experimenters simultaneously present to each focal female two males belonging to distinct categories and record which male succeeds in mating. An alternative protocol is to present each focal female with only a single male at a time and record mating rates and latencies. This protocol, often referred to as 'no-choice', prevents females from comparing males with distinct features before deciding whether to mate or not with the only male they have encountered. Choice protocols reveal stronger mate preferences than do no-choice tests perhaps owing to the ease of comparison between prospective mates and lower cost of rejecting a potential mate when females encounter two rather than one male (Dougherty & Shuker, 2015). The choice protocol, however, cannot rule out male–male interactions, which could affect access to females even in species with no overt aggression.

The recent adoption of fruit flies as a model system for mechanistic research on aggression (Asahina, 2017; Chen, Lee, Bowens, Huber, & Kravitz, 2002) has sensitized us to the possibility that apparent mate choice under the prevalent choice protocol is influenced by subtle male aggression. As with many other species (Emlen & Oring, 1977), fruit flies' mating system varies as a function of density and resource distribution. The recent work on fruit fly aggression has appropriately focused on the mating system scenario characterizing low fly density and small, defensible fruits. In such settings, fruit flies show resource defence polygyny, where capable males rely on aggression to defend attractive fruits where females gather to feed and lay eggs (Baxter, Barnett, & Dukas, 2015b; Hoffmann & Cacoyianni, 1990; Markow, 1988). Under the prevalent conditions of high fly density and large food clumps, however, the predominant assertion has been that male fruit flies show scramble competition for mates (Spieth, 1974), a mating system that is rather common in insects (Thornhill & Alcock, 1983). Scramble competition is consistent with choice protocols, because it assumes that multiple males court females and females choose their mates.

Because past observations in many laboratories including ours indicated no overt aggression during mate choice trials, we wished to test whether subtle aggression could bias male access to females. Based on previous work, we chose two realistic choice scenarios, one involving males that varied in age and the other involving males that varied in size. For both scenarios, our own and others' research suggested female preference for older over younger males (Baxter, Barnett, & Dukas, 2015a; Dukas & Baxter, 2014; Long, Markow, & Yaeger, 1980) and for large over small males (Dukas, 2005; Partridge, Ewing, & Chandler, 1987). These studies, however, did not assess the possible influence of subtle male aggression. We thus asked whether males rely on subtle aggression to monopolize access to females, and whether such interactions bias male mating success. To this end, we first conducted video recording trials, each with two males and an immature female to quantify subtle aggression. Second, we simultaneously assessed male mating success in choice and no-choice trials. Finally, to resolve the weaknesses of the no-choice and choice trials, we developed a new protocol in which a female can freely assess sequentially two males housed in distinct compartments and then choose her mate. We refer to this set up as 'true-choice'. We then compared male mating success under the classical choice protocol, which cannot separate effects of male–male interactions and female choice, and under the true-choice set-up, which eliminates male–male interactions.

GENERAL METHODS

We used descendants of wild-caught *D. melanogaster* collected in several southern Ontario localities in August 2014. We housed the flies in population cages containing several hundred flies per cage. We kept the cages in an environmental chamber at 25 °C and 60% relative humidity with a 12:12 h light:dark cycle, with the lights turning on at 1000 hours. Unless stated otherwise, we reared the experimental flies at a low density of about 300 eggs per 240 ml bottle containing 50 ml of standard fly medium made of water, sucrose, cornmeal, yeast, agar and methyl paraben. We sexed flies within 4 h of eclosion to ensure virginity and minimal experience with other flies. We used gentle aspiration to live-sex and transfer males into individual 40 ml vials each containing 5 ml of fly medium. We anaesthetized females with CO₂ to sex and place them in groups of 20 per vial, which contained the same amount of fly medium plus a dash of live yeast.

Unless stated otherwise, we conducted all tests in cylindrical arenas made of Plexiglas (3 cm in diameter and 2.5 cm high). We covered the floor of each arena with a piece of filter paper. To deter flies from climbing on the arenas' walls and ceilings, we coated the walls with Insect-a-Slip (Fluon; BioQuip, Gardena, CA, U.S.A.) and the ceilings with Surfasil (Sigma Aldrich, Oakville, ON, Canada). When dry, Fluon and Surfasil form an odourless slippery film, which flies cannot walk on. Fluon and Surfasil have been used in insect studies for a long time and do not appear to have negative effects (Asahina et al., 2014; Radinovskiy & Krantz, 1962).

MALE SIZE, COURTSHIP INTERFERENCE AND MATING SUCCESS

We first wished to test whether males relied on subtle aggression for monopolizing access to females. To this end, we relied on close-up video recordings to quantify subtle aggression between small and large males in choice trials. Based on preliminary observations, we focused on male take-overs, defined as a male positioning himself between the courting male and the female and taking over the courtship (Fig. 1a). Previous data indicated that large males outcompete small males for access to attractive food patches (Asahina, 2017; Hoffmann, 1987). We thus predicted higher take-over rates by large males than by small males. To assess the effects of courtship interference on male courtship, we compared the courtship duration of large and small males under both no-choice and choice protocols (Fig. 1b). We predicted that large males would spend more time courting females in the choice trials than in the no-choice trials than would small males. Finally, we predicted a higher relative mating success by large males than by small males in the choice trials than in the no-choice trials.

Courtship Interference

Methods

We conducted two types of trials: no-choice and choice (Fig. 1a). In no-choice trials, we placed one male (either a small or large male) with one immature virgin female (<18 h post eclosion) in an arena ($N_{\text{small}} = 30$, $N_{\text{large}} = 30$). In the choice trials, we placed two males (one small and one large male) with one immature female in an arena simultaneously ($N_{\text{choice}} = 30$). We used immature virgin females because they are as sexually attractive to males as mature virgin females, but they always reject males (Dukas & Dukas, 2012). By using females who consistently reject males we could accurately assess male courtship and competitive ability, without female preference affecting each male's courtship opportunities.

We obtained small and large males by rearing flies under different densities. While we refer to the males by their obvious size differences, they probably differed in a variety of other traits

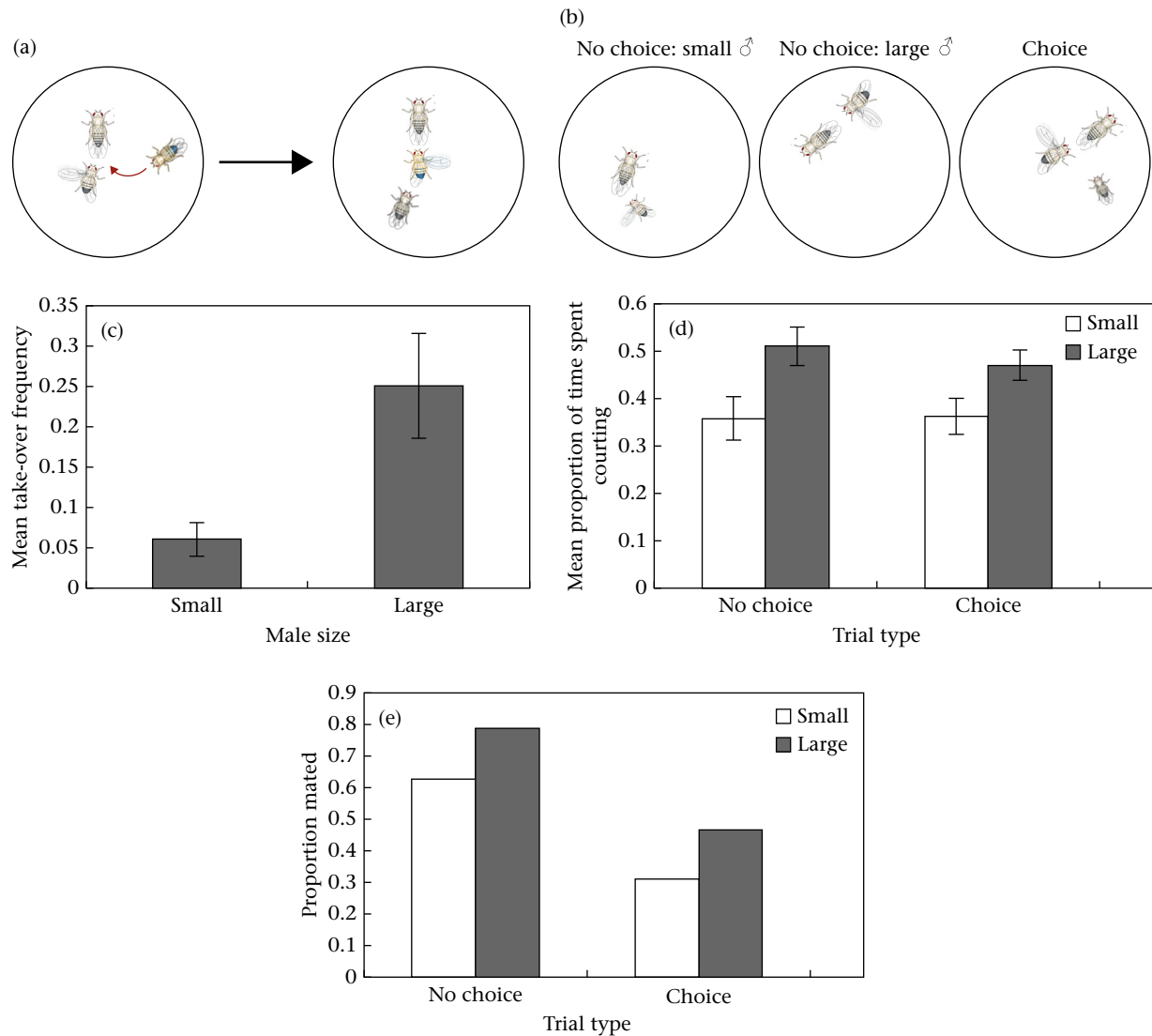


Figure 1. (a) Depiction of a courtship take-over. In the left circle, male 1 is courting the female and male 2 pushes in between them. In the right circle, male 2 has displaced male 1 and is courting the female. (b) Diagram of the protocols for courtship interference and mating success in small versus large males. Females were sexually immature in the courtship experiment and sexually mature in the competitive mating experiment. Note that males are smaller than females, and have a dark posterior. (c) Mean \pm SE take-over frequency for small and large males in the choice trials ($N_{\text{choice}} = 30$). (d) Mean \pm SE proportion of time males spent courting a female in no-choice trials (1 male + 1 female ($N_{\text{small}} = 30$, $N_{\text{large}} = 30$)) and in choice trials (small male + large male + female simultaneously ($N_{\text{choice}} = 30$)). (e) Proportion of small and large males that mated in no-choice ($N_{\text{small}} = 123$, $N_{\text{large}} = 122$) and choice trials ($N_{\text{choice}} = 122$).

influenced by larval density (Bangham, Chapman, & Partridge, 2002; Lüpold, Manier, Ala-Honkola, Belote, & Pitnick, 2011; Wigby, Perry, Kim, & Sirot, 2016). We reared small males in high-density food vials with approximately 200 eggs per vial containing 1.5 ml of the standard fly medium, whereas we reared large males in low-density food bottles with approximately 100 eggs per bottle containing 50 ml of the standard fly medium. Within 4 h of eclosion, we transferred adult males to individual vials with ample food until the time of testing (4 days later). Females were reared in food bottles with 50 ml of food and 300–400 flies per bottle. After testing, we measured the wing length of a random sample of 12 small and 12 large males. We removed the right wing of each male, mounted it on a microscope slide and measured the linear distance between the humeral–costal break and the end of the third longitudinal vein (Gilchrist & Partridge, 1999). The wings of large males were approximately 25% longer than those of small males, 1.85 ± 0.017 versus 1.48 ± 0.020 mm, respectively (t test: $t_{22} = 14.14$, $N = 24$, $P < 0.001$).

After placing the flies in the arena, we began videorecording for 15 min using Logitech HD Pro C920 and iPad Air cameras. Observers scored the videos using the Observer software (Noldus Information Technology, Wageningen, The Netherlands). Due to the males' visible size differences, it was not possible for observers to be blind to the male size manipulation. However, the observers were not aware of our predictions about male size and competitive courtship ability.

In the no-choice condition, where there was just one male and one female per arena, observers recorded the duration of courtship, and who terminated each courtship bout. If the male turned or flew away from the female, or simply stopped chasing her, we counted it as the male terminating the courtship bout. If the female decamped (jumped or flew away from the male), we counted it as the female terminating the courtship bout (Dukas & Scott, 2015). In the choice condition, where there were two males and one female per arena, observers recorded the duration of courtship and the number of courtship take-overs performed by each male. Because courtship

typically involves the male closely following the female, only one male can court at any given time. Furthermore, only the non courting male can interfere with the courtship of the other male. That is, courtship and courtship interference are mutually exclusive.

We used linear and generalized linear mixed-effects models (LMM and GLMM) in R v.3.3.3 (R Core Team, 2014) with the package lme4 v.1.1–12 (Bates, Maechler, Bolker, & Walker, 2014) to analyse the take-over frequency, courtship duration and courtship termination data. For tests of the fixed effects, we report Wald χ^2 values generated with the Anova function from the car package v.2.1–4 (Fox & Weisberg, 2011). For the take-over data, we used a GLMM with a negative binomial distribution with the total number of take-overs each male performed as the dependent measure, male size as a fixed factor and arena identity as a random factor (to account for the nonindependence of two males in the same arena). We also included the log duration of each male's competitor's courtship as an offset in the model to account for the fact that a male's take-over opportunities are constrained by his competitor's courtship (for example, a male whose competitor barely courts has fewer take-over opportunities than a male whose competitor courts frequently). For the courtship duration data, we used an LMM with the proportion of time spent courting as the dependent measure, male size and trial type as fixed factors and arena identity as a random factor. We analysed the female courtship termination data similarly to the take-over data (with total female courtship terminations as the dependent measure and the log duration of courtship as an offset).

Results

In the choice trials, large males performed significantly more courtship take-overs than small males (GLMM with negative binomial distribution: $\chi^2_1 = 14.9$, $N = 60$, $P < 0.001$; Fig. 1c). Across both trial types, large males spent significantly more time courting than small males (LMM: $\chi^2_1 = 7.67$, $N = 90$, $P < 0.01$; Fig. 1d). However, contrary to our prediction, the effect of the interaction between male size and trial type on the duration of time males spent courting was not significant ($\chi^2_1 = 0.42$, $N = 90$, $P = 0.52$; Fig. 1d). In the no-choice trials, we recorded who terminated each courtship bout (i.e. the female or the male). There was no effect of male size on the frequency with which females terminated courtship (GLMM with negative binomial distribution: $\chi^2_1 = 0.045$, $N = 60$, $P = 0.83$).

Mating Success in Choice Versus No-Choice Trials

Methods

In the previous experiment, we used sexually immature females so that we could assess male courtship effort and competitive ability under controlled conditions of consistent rejection from females. To assess the differential mating success of males with and without competition, we performed a similar experiment to the previous one, but used mature virgin females.

We again conducted no-choice and choice trials (Fig. 1a). In the no-choice trials, we placed one male (small or large) with a mature, 4-day-old virgin female in the arena ($N_{\text{small}} = 123$, $N_{\text{large}} = 122$). In the choice trials, we placed two males (one small and one large) with a mature, 4-day-old virgin female in the arena ($N_{\text{choice}} = 122$). Observers who were naïve to our predictions scanned the arenas for mating. Trials ended once mating occurred, or after 30 min had passed.

As in the previous experiment, after testing, we measured the wing length of a random sample of 13 small and 13 large males. The wings of large males were approximately 24% longer than those of

small males, 1.80 ± 0.014 versus 1.45 ± 0.014 mm, respectively (t test: $t_{24} = 17.31$, $N = 26$, $P < 0.001$).

We performed a binomial test in SPSS v.22 (IBM Corp., Armonk, NY, U.S.A.) to compare the observed proportions of matings in the choice treatment to the expected proportions, which were calculated based on the mating success of small and large males in the no-choice treatment. In this analysis, we treated each vial containing two males and a female as the experimental unit.

Results

The proportion of large males that mated in the choice trials was nearly identical to what was expected based on the proportion that mated in the no-choice trials (binomial test: $N_{\text{no-choice}} = 245$, $N_{\text{choice}} = 122$, $P = 0.24$; Fig. 1e).

MALE AGE, COURTSHIP INTERFERENCE AND MATING SUCCESS

This set of experiments was analogous to the set of experiments above except that males varied by age rather than size (Fig. 2a). We used males that were 1 and 4 days old, as males of both of these ages are sexually mature and readily court and mate with females (Baxter & Dukas, 2017; Baxter et al., 2015a; Dukas & Baxter, 2014). First, we predicted that 4-day-old males (hereafter mature males) would spend more time courting females than 1-day-old males (hereafter young males) in the choice than in the no-choice trials. Second, we predicted higher take-over rates by mature males than by young males. Finally, we predicted a higher relative mating success by mature males than by young males in the choice trials than in the no-choice trials.

Courtship Interference

Methods

In no-choice trials, we placed either a young or mature male with an immature virgin female ($N_{\text{young}} = 28$, $N_{\text{mature}} = 30$). In the choice trials, we placed one young and one mature male with an immature female in the arena simultaneously ($N_{\text{choice}} = 58$). To differentiate between males in the choice treatment, we coloured males with pink and blue fluorescent powder while counterbalancing male colour with male age.

After placing the flies in the arena, we began videorecording for 15 min. Later, observers blind to male age scored the videos. In the no-choice condition, observers recorded the duration of courtship, and who terminated each courtship bout. In the choice condition, observers recorded the duration of courtship and the number of courtship take-overs performed by each male.

Results

In the choice trials, mature males performed significantly more courtship take-overs than young males (GLMM with negative binomial distribution: $\chi^2_1 = 15.6$, $N = 58$, $P < 0.001$; Fig. 2b). Across both trial types, mature males spent significantly more time courting than young males (GLMM with gamma distribution and inverse link function: $\chi^2_1 = 12.4$, $N = 116$, $P < 0.001$; Fig. 2c). There was a significant interaction between male age and trial type, meaning that there was a larger difference between the courtship duration of mature males versus young males in the choice trials than in the no-choice trials ($\chi^2_1 = 6.06$, $N = 116$, $P < 0.05$; Fig. 2c). That is, mature males had a competitive courtship advantage over young males.

In the no-choice trials, females terminated courtship with mature males more frequently than they terminated courtship with young males, but this difference did not reach significance (GLMM with negative binomial distribution: $\chi^2_1 = 2.41$, $N = 58$, $P = 0.12$).

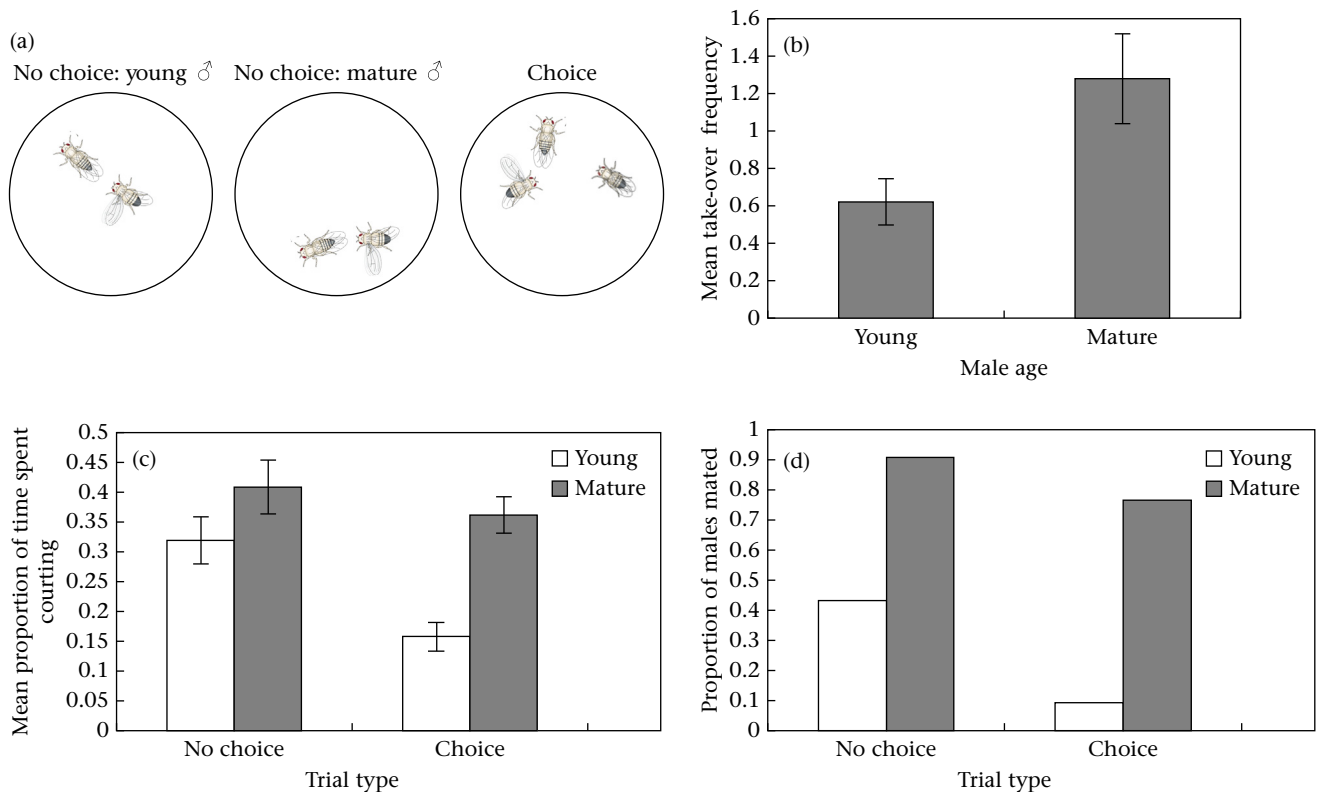


Figure 2. (a) Diagram of the protocols for young versus mature males' courtship interference and mating success. In the courtship experiment, the females were sexually immature, whereas in the competitive mating experiment, they were sexually mature. (b) Mean \pm SE take-over frequency for young and mature males in the choice trials ($N_{\text{choice}} = 58$). (c) Mean \pm SE proportion of time males spent courting a female in no-choice ($N_{1\text{-day-old}} = 28$, $N_{4\text{-day-old}} = 30$) and choice trials ($N_{\text{choice}} = 58$). (d) Proportion of young and mature males that mated in no-choice ($N_{1\text{-day-old}} = 120$, $N_{4\text{-day-old}} = 120$) and choice trials ($N_{\text{choice}} = 120$).

Mating Success in Choice Versus No-Choice Trials

Methods

In the previous experiment, we used sexually immature females to assess male courtship effort and competitive ability. To assess how competitive courtship ability translates into mating success, we repeated the same experiment with mature virgin females, again conducting no-choice and choice trials (Fig. 2a). In the no-choice trials, we placed one male (young or mature) with a mature virgin female (4 days old) in the arena ($N_{\text{young}} = 120$, $N_{\text{mature}} = 120$). In the choice trials we placed two males (one young and one mature) with a mature virgin female in the arena ($N_{\text{choice}} = 120$). Observers blind to male age scanned the arenas for matings. Trials ended once mating occurred, or after 30 min had passed.

We used a binomial test to compare the observed proportions of matings in the choice treatment to the expected proportions, which were calculated based on mating success in the no-choice treatment by young and mature males (SPSS v.22, IBM Corp.).

Results

The proportion of mature males that mated in the choice trials was significantly larger than that expected from the no-choice trials (binomial test: $N_{\text{no-choice}} = 240$, $N_{\text{choice}} = 120$, $P < 0.001$; Fig. 2d).

TRUE-CHOICE VERSUS CLASSICAL CHOICE

The experiments above indicated that subtle interactions between males could determine the outcomes of mate choice tests. That is, data attributed to female mate choice under the classical

choice protocol can actually be driven by male–male competition for access to females. There is thus an obvious need for an additional protocol that eliminates male–male competition from the choice protocol. To address this requirement, we developed a new apparatus that allows a female to freely travel between two compartments, each housing one male, and then choose her mate. We refer to this set-up as 'true-choice'. We then compared the mating success of young and mature males under the choice protocol and under the true-choice set-up. Based on the data above (Fig. 2), we expected higher relative mating success by mature males than by young males in the choice trials than in the true-choice trials.

Methods

We constructed two types of arenas for the true-choice and choice trials (Fig. 3a). The true-choice arena was a novel apparatus inspired by Byrne, Rice, and Rice (2008). It had two adjacent rectangular compartments that isolated the males from each other. Each compartment was $1 \times 1 \times 4$ cm in size. In the shared wall of the two compartments were 24 holes approximately 0.95 mm in diameter. The holes were small enough to prevent large flies (in this case males) from passing between compartments, but at the same time large enough to allow small flies (in this case small females) to pass through and visit both compartments. The choice arena, which allowed for simultaneous female choice and male–male competition, consisted of a single compartment $1 \times 1 \times 6.5$ cm in size. Both arenas contained standard food to the 1.5 cm mark from one end and sealed with a foam plug at the other. After adding food, the space in the choice arena was twice the length of the true-choice

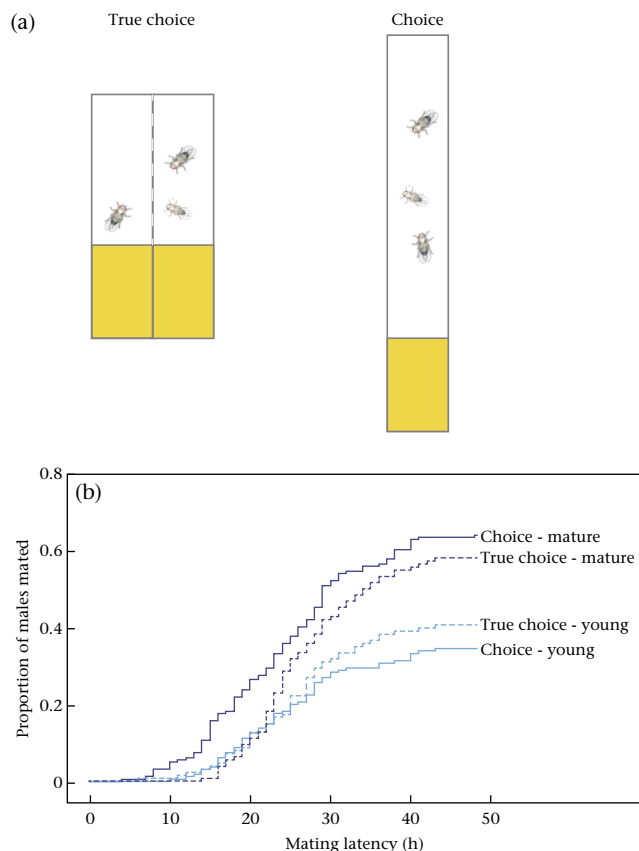


Figure 3. (a) Diagram of the protocol for choice versus true-choice treatments. Note that males have a dark posterior, but, due to the experimental manipulation, they are larger than females. (b) Proportion of matings by young and mature males over time in the choice ($N = 157$) and true-choice ($N = 124$) treatments.

arena so that the total space the female could explore in both arenas was equivalent.

In fruit flies, females are typically slightly larger than males. To develop small females, we reared them at a density of approximately 250 eggs per 1 ml of standard fly medium (Baxter et al., 2015a; Byrne et al., 2008). Although small females are less attractive than large females as shown by less courtship from males, young and mature males court small females with equal intensity (Baxter et al., 2015a). To develop large males, we reared them at a density of 100 eggs per 50 ml of medium.

We wanted females to have ample time to interact with each male before choosing to mate with one of them. To this end, we placed sexually immature, recently eclosed females in the arenas with a recently eclosed male and a 3-day-old male. This allowed the females to have an extended period of interaction with both males before becoming sexually mature, approximately 12 h later. Once females became sexually mature, they were able to make informed choices of whether to mate with the now 1-day-old (young) or 4-day-old (mature) male. In the true-choice arena, females started half of the trials with the mature males and half the trials with the young males, and this was counterbalanced with her starting on the left or right side of the arena.

We ran trials in humidified chambers at 25 °C and $80 \pm 10\%$ relative humidity with bright ambient light. Trials lasted 48 h beginning when we placed flies into the arenas. We recorded the arenas with iPods using the time-lapse application OhSnap! to record a single frame every 3 min. Observers blind to male age scored the first mating of each female from the resulting time-lapse photos. A mating was counted if the same male was mounting a

female for three to five consecutive frames. This was an adequate measure as matings in *D. melanogaster* typically last for about 15 min (Ashburner, 1989).

We excluded from the data two types of trials. First, we removed true-choice trials where females did not receive courtship by both males before mating ($N = 16$) as this did not constitute a choice by the female. Second, we excluded all trials that did not result in mating ($N_{\text{choice}} = 11$, $N_{\text{true-choice}} = 7$). This left us with a sample size of 157 choice trials and 124 true-choice trials. We split the trials into six 8 h blocks to assess the proportion of matings in each trial type across time. We analysed the data in R (R Core Team., 2014) using the gee package v.4.13–19 (Carey, Lumley, & Ripley, 2015). We used a generalized estimating equation (GEE) with a binomial distribution to assess the cumulative proportion of matings attained by mature males across the 8 h blocks, with arena identity as the subject identity (for repeated samplings of the same arena across the 8 h blocks) and 8 h block as an ordered factor. We predicted that the opportunity for male–male interactions would result in a larger proportion of matings attained by the mature males in the choice trials than in the true-choice trials.

Results

The mature males had a higher proportion of matings in the choice treatment than in the true-choice treatment (GEE with binomial distribution: robust $z = 2.15$, $N = 281$, $P < 0.05$; Fig. 3b).

DISCUSSION

We documented robust but subtle aggressive interactions between males that affected their ability to court females: males persistently interfered with each other's courtship, attempting and often succeeding in taking over courtship from another male (Fig. 1a). That is, we believe that the classical characterization of fruit fly mating system under high density as scramble competition (Partridge et al., 1987; Spieth, 1974) should be revised to interference competition. Furthermore, we think that careful observations will necessitate reclassifying many other apparent scramble competition cases (Shuker & Simmons, 2014; Thornhill & Alcock, 1983) as interference competition. In fruit flies, we and others had failed to notice courtship take-over in many previous experiments using a choice protocol, which, in spite of its name, cannot distinguish between choice by females and subtle male–male competition. To detect the courtship take-overs, we required close-up video recordings. Once we established the protocol for measuring courtship take-overs, we could quantify highly replicable patterns of large males performing more take-overs than small males (Fig. 1c), and of mature males performing more take-overs than young males (Fig. 2b). Our results are consistent with data on fruit fly aggression in the context of resource defence polygyny, where large males outcompete small males (Asahina, 2017; Hoffmann, 1987) and mature males win over young males (Baxter & Dukas, 2017).

Courtship interference is known in a variety of taxa (Wong & Candolin, 2005). For example, although females appear to choose among males occupying a lek, courtship interruption is prevalent (Foster, 1983). In the Guianan cock of the rock, *Rupicola rupicola*, males that disrupted other males' courtship or copulation had a higher mating success, and males that engaged in more aggressive and persistent interference were more likely to mate with the females they interrupted (Trail, 1985). In the water boatman *Sigara falleni*, males frequently engaged in overt aggression, interrupting courtship and mounting attempts. In choice trials dominated by male courtship interference, large males with smaller palae for their size had the highest mating success, while in no-choice trials,

large males with large relative palae had the highest mating rates (Candolin & Tregenza, 2004). Finally, in the Pacific blue-eye, *Pseudomugil signifier*, courtship bouts under no-choice were much longer than under choice trials. While dominant and subordinate males were equally likely to disrupt each other's courtship, dominant males monopolized courtship in the choice trials (Wong, 2004). An important difference between our findings and previous reports on courtship interference is that we have documented subtle courtship interference, which could be revealed only through careful analyses of video recordings. Such covert male–male interactions may be prevalent, with their effects on sexual selection underappreciated.

Our three sets of experiments revealed a complex pattern, which most likely reflects the actual intricacy of the dynamics that determine mating success. In a choice protocol involving small and large males and an immature female, large males took over courtship (Fig. 1a) four times more often than small males (Fig. 1c). Remarkably, however, in spite of the persistent interference from large males, the small males were determined at resuming courting females. This is reflected in the fact that, in the no-choice trials, females received courtship during less than half of the trial duration (the average of the two left bars in Fig. 1d) compared to about 0.85 of the trial duration in the choice trials (the sum of the two right bars in Fig. 1d). Thus the persistence of the small males resulted in no relative loss in mating success in the choice trials compared to the no-choice trials (Fig. 1e). This indicates that there is no simple positive correlation between courtship interference and either the proportion of time spent courting, or mating success, at least not under our controlled experimental conditions.

The outcomes of matches between young and mature males were different from those of the small and large males (Fig. 2). Here mature males took over courtship twice as often as young males (Fig. 2b) and this resulted in the mature males spending over twice as much time as young males courting females under the choice conditions compared to only a small courtship bias under no-choice (Fig. 2c). Moreover, courtship interference translated into a significant mating advantage for mature males over young males in the choice setting than in the no-choice setting (Fig. 2e). Note that, because we also recorded females' frequencies of terminating courtship, we know that females did not decamp more often when courted by small versus large males or young versus mature males. There was even a marginally significant tendency for females to decamp more often when courted by mature males than when courted by young males.

The most likely explanation for the distinct effects of courtship interference on the relative mating success of small and young males (Fig. 1e versus Fig. 2d) is the age difference between the males. Our previous work on the life history of aggression indicated that, compared to young males, mature males that have been deprived of females show a higher motivation to gain matings, which translates into higher levels of aggression in male–male contexts over resources and greater tendencies by males to persistently pursue females with low receptivity and to engage in forced copulations (Baxter & Dukas, 2017). It is likely that the small, mature males responded to courtship interference by persistently resuming courtship when the large males discontinued their female pursuit, whereas the young males responded to the more aggressive mature males by retreating.

Finally, although our data revealed robust, subtle male contest and suggested that it could influence male mating success in the context of the female choice protocol, the data did not show a direct effect of courtship interference on mating success. To test for such direct influence, we had to fully separate male contest from female choice, a requirement that the traditional choice and no-choice

protocols do not accomplish. To this end, we developed a novel protocol, true-choice, which met that requirement (Fig. 3a). Using the true-choice protocol, we found that male–male interactions indeed affected mating success (Fig. 3b). We think that true-choice protocols should replace choice protocols in future studies assessing traits determining mating success in species where physical contact is essential for courting and mate assessment.

We noted some difficulties with the true-choice protocol. Male harassment of females is prevalent in settings where females cannot escape pursuing males (Baxter & Dukas, 2017; Dukas & Jongsma, 2012). We thus think that the perfect true-choice protocol should provide females with the option of evading males. Our preferred prototype for the true-choice apparatus had a central female compartment and two side compartments for each male, as this design provides females with a male-free shelter. The weakness of that apparatus, however, was that we had to drop a high proportion of trials in which females mated prior to assessing both males. Another possible concern is the fact that the true-choice protocol required us to reverse the typical pattern of females being larger than males. Such size reversal, however, may occur in nature in cases where some females have a high-density larval environment while some males happen to develop at low density. We and others have successfully used small females previously. While males find small females less attractive than large females as indicated by lesser courtship and mating preference (Baxter et al., 2015a; Byrne & Rice, 2006), both small-female behaviour and males' responses to them seem natural.

We think that our data indicating covert courtship interference should be considered in future mate choice studies. Distinguishing between female choice and male–male interactions is important only if there is a conflict between the sexes such that traits preferred by females are distinct from traits selected through male–male contests (Arnqvist & Rowe, 2005; Qvarnström & Forsgren, 1998). For example, if both male competition and female choice select for larger body size as is the case in many studies (Hunt et al., 2009), then separating the mechanisms may not be crucial. It is likely, however, that research focusing on sexually selected traits utilized by males and females under realistic settings will reveal distinct roles of traits affecting male competition and female choice. While such work has been encouraged in the past (Hunt et al., 2009; Lande & Arnold, 1983), we still lack sufficient data. We suggest that future research on the topic employ true female choice protocols, which allow for female assessment of males and choice while fully eliminating male competition.

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