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 intersexual 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; Wlodarski & 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 presented 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, defendable 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 CO_2 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; Radinovsky & 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 ($N_{small} = 30$, $N_{large} = 30$). In the choice trials, we placed two males (one small and one large male) with one immature female in an arena simultaneously ($N_{choice} = 30$). 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