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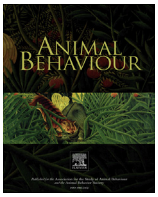

The adaptive role of a species-specific courtship behaviour in coping with remating suppression of mated females

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Three-way interactions consisting of a female and her current and previous mates have been studied intensively in the context of sperm competition involving male manipulation of female remating rate but have rarely been documented in a broader context involving classical premating male display and stimulatory traits such as courtship behaviour. This is surprising because premating traits influence the intensity of postmating competition, which occurs only when a mated female consecutively accepts another male. In *Drosophila* fruit flies, the subsequent male has an advantage over the previous male in sperm competition. However, the ejaculate of the previous male changes the female's behaviour to refuse remating for several days (remating suppression), reducing the potential advantage of the subsequent male. Under such conditions, the evolution of any means that counteract remating suppression is thought to be adaptive for the subsequent male. Males of the fruit fly *Drosophila prolongata* perform a unique courtship behaviour called ‘leg vibration’. Although leg vibration increases female receptivity, it is not always required for mating with virgin females, raising a question of why it evolved in the first place. In this study, the role of leg vibration in remating was examined, using leg amputation to manipulate the efficiency of leg vibration and an eye colour mutation to detect remating events. Leg vibration had a profound effect on mated females: the remating rate was extremely low with leg-amputated males, indicating that leg vibration was almost indispensable for remating of recently mated females. Our results demonstrated that single courtship behaviour has different levels of importance or necessity for the first male and the subsequent males, providing an example of the evolution of courtship behaviour that was possibly driven by postmating competition.

Whether a mated female remates with other males is one of the most important aspects of animal reproductive systems, affecting the strength of sexual selection on males (Kvarnemo & Ahnesjö, 1996; Markow, 2002; Shuker & Simmons, 2014). If the remating rate of females is low, for example, males are forced to compete for limited opportunities to mate with virgin females. Under such conditions, the evolution of the male's ability to induce remating of an already mated female should be adaptive by freeing the male from relentless competition for scarce virgin females (Gavrilets & Hayashi, 2006; Markow, 2002).

The male's ability to induce remating, however, is confronted with counter adaptation by females. The theory of sexual conflict predicts that, even in systems where females usually mate multiple times with different males (polyandry), females evolve to refuse an excess number of costly matings, which counteracts the male's ability to induce remating (Arnqvist & Rowe, 2005; Gavrilets, Arnqvist, & Friberg, 2001). Therefore, regardless of the mating system (monandry or polyandry), males are challenged by females that refuse remating, which acts as a continuous selection pressure for further evolution of the male's ability to induce remating (Holland & Rice, 1998).

The male's ability to induce remating is also confronted with counter adaptation by other males. Although remating of mated females may relax precopulatory selection by increasing the total mating opportunities, it provokes another type of male–male competition: sperm competition (Birkhead & Møller, 1998; Kvarnemo & Simmons, 2013; Parker, 1970). Sperm of the subsequently mated male competes with that of the previous male for fertilization, reducing the paternity share of the previous male. Even worse for the previous male, the last male sires most of the progeny in some taxonomic groups (Birkhead & Møller, 1992; Simmons, 2001; Smith, 1984). This competition is further facilitated by cryptic female choice (Eberhard, 1996; Firman, Gasparini, Manier, & Pizzari, 2017). In both cases, remating of a mated female reduces the fitness of the previous male. Therefore, males are expected to evolve means to manipulate females to refuse remating (Gavrilets & Hayashi, 2006).

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In fruit flies of the *Drosophila melanogaster* species group, it is well established that the female remating rate is suppressed for several days after the first mating (Connolly & Cook, 1973; Manier et al., 2013; Markow, 2002). This remating suppression is mediated by seminal fluid proteins such as sex peptide (SP) expressed in the male accessory reproductive gland and transferred to females during copulation (Aigaki, Fleischmann, Chen, & Kubli, 1991; Chapman et al., 2003; Chen et al., 1988; Liu & Kubli, 2003; Wolfner, 1997). Because of the last male sperm precedence in the *melanogaster* species group (Manier et al., 2010, 2013; Price, 1997; Price, Dyer, & Coyne, 1999), remating suppression has been thought to be a male manipulation of females to protect paternity (Fricke, Wigby, Hobbs, & Chapman, 2009; Smith, Clarke, Boone, Fricke, & Chapman, 2017). However, recent findings on the SP receptor and dedicated neural circuits in females mediating the response to SP imply that evolution on the female side is also involved in remating suppression (Feng, Palfreyman, Häsemeyer, Talsma, & Dickson, 2014; Tsuda & Aigaki, 2016; Tsuda, Peyre, Asano, & Aigaki, 2015; Yapici, Kim, Ribeiro, & Dickson, 2008). In other words, it is possible that females exploit SP as a cue to suppress receptivity, thus avoiding an excess number of costly rematings. Although it is still under debate whether females of the *melanogaster* species group gain or lose fitness by remating suppression, it obviously favours the previous male by nullifying the potential advantage of the subsequent male in sperm competition.

Under such conditions, the male's ability to induce remating of mated females may face different selective pressures compared with the ability to mate with virgin females. In other words, a given courtship behaviour could have a different degree of importance or necessity in mating with virgin females and mated females. For example, more elaborate courtship behaviour may be required for mating with recently mated females than mating with virgin females.

Drosophila prolongata, a member of the *rhopaloa* subgroup of the *melanogaster* species group, has unique morphology and mating behaviour (Setoguchi et al., 2014; Singh & Gupta, 1977; Toda, 1991). The forelegs of male *D. prolongata* are extraordinarily thick and elongated. In addition to wing vibration, which is a common courtship behaviour observed in many *Drosophila* species, courtship in *D. prolongata* males involves a unique behaviour called ‘leg vibration’, in which males hit the female's body using their forelegs from in front of her (Fig. A1, Supplementary video 1; Setoguchi et al., 2014). Leg vibration increases copulation receptivity of virgin females and is frequently observed prior to copulation attempts (Setoguchi, Kudo, Takanashi, Ishikawa, & Matsuo, 2015). Because elongated forelegs are a prerequisite to perform leg vibration, the morphology and the behaviour are tightly linked to each other. These characters are not observed in the other *Drosophila* fruit flies, suggesting that they have specifically evolved in the *D. prolongata* lineage (Setoguchi et al., 2014). Note, however, that not all virgin females (30–70%) require leg vibration to accept copulation (Kudo, Takamori, Watabe, Ishikawa, & Matsuo, 2015), and males do not always perform leg vibration before attempting copulation, particularly in the presence of rivals that may intercept females (10–70%; Setoguchi et al., 2015). These results show that leg vibration is dispensable in the courtship of *D. prolongata*. This is paradoxical, considering the foreleg morphology that looks as if it evolved for its dedicated function in leg vibration. However, because all experiments to date were conducted using virgin females, the role of leg vibration in remating has not been explored.

Supplementary video related to this article can be found at https://doi.org/10.1016/j.anbehav.2018.04.002.

In this study, the effect of leg vibration on mated females was examined. Efficiency of leg vibration was manipulated by amputation of the male legs. Successful remating events were detected by determining the paternity of offspring using an eye colour mutation. These two techniques enabled us to test the hypothesis that leg vibration is adaptive for the subsequent males, by examining the following conditions. (1) The second male has an advantage over the first male in terms of sperm competition (last male sperm precedence; experiment 1). (2) Receptivity of mated females is lower than that of virgin females (remating suppression: experiment 2). (3) Leg vibration is more important for mating with mated females than virgin females (experiment 3).

METHODS

Fly Strains

A previously described strain, SaPa010 (Kudo et al., 2015), was used as wild type in this study. This strain was established from a single mated female collected in 2004 at Sa Pa, Vietnam (22°20′N, 103°52′E), and has been maintained for more than 100 generations by inbreeding under laboratory conditions with an average population size of around 50 individuals in a culture bottle (Drosophila bottles AS355, Thermo Fisher Scientific, Waltham, MA, U.S.A.). To identify paternity in the progeny produced by females that mated multiple times, an eye colour mutation, *vermilion*$^-$, was used. The mutation was originally generated in the genetic background of another strain, BaVi044, by *N*-ethyl-*N*-nitrosourea (ENU) mutagenesis (Kudo, Awasaki, Ishikawa, & Matsuo, 2018). The *vermilion* locus was sex linked, and hemizygous males and homozygous females were readily distinguishable by their bright eye colour. To introduce the *vermilion*$^-$ mutation into the SaPa010 genetic background, the original mutant strain was backcrossed to SaPa010 for five cycles (one cycle consists of two generations: a phenotype-screening generation in males and a recombination generation in females), to obtain the *vermilion*$^-$ strain used in this study. Flies were reared on standard cornmeal medium for *Drosophila* culture (Setoguchi et al., 2014). All cultures were maintained at 20 °C and all experiments were carried out at the same temperature because development of *D. prolongata* is inhibited at higher temperatures (Hitoshi, Ishikawa, & Matsuo, 2016a). A 12:12 h light:dark cycle was applied throughout the experiments. Under this condition, the developmental period required from egg to adult was 16 days for females and 18 days for males, and the life span was at least 1 month for both sexes.

Preparation of Females

Newly eclosed female flies (within 24 h) were maintained in vials containing culture medium for 5 days in a group of up to 10 individuals per vial (Fig. 1a). Prior to the first mating treatment on the 6th day after eclosion, flies were starved for 1 day in vials containing a wet cotton ball. This starvation treatment increases the locomotor activity during the mating experiments, and was used in most of our previous experiments (Hitoshi, Ishikawa, & Matsuo, 2016b; Kudo, Shigenobu et al., 2017; Kudo et al., 2015; Matsuo, 2018; Setoguchi et al., 2015). On the following day, each female was paired with a 7-day-old male (prepared as described below) in a medium-containing vial for 1 h (3–2 h before the start of the dark phase). Copulation was confirmed by direct observation, and mated females were recovered into a vial with a wet cotton ball and starved for another day. These flies were used in the remating experiment on the 7th day. Few females laid eggs between the first and second matings. Although this is not likely to occur in the wild, it allowed us to precisely examine the full effect of the first male's ejaculates on sperm precedence and copulation rate. Virgin females were prepared in the same way except for the first mating treatment which was carried out without males. The *vermilion*$^-$ females were used for analysis of sperm precedence (experiment 1) and