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Aggression, mate guarding and fitness in male fruit flies

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Aggression is a central trait affecting fitness, which has been well studied in many animals. As a part of a research programme integrating mechanisms and fitness consequences of aggression, we examined the adaptive functions of antagonistic interactions in fruit flies, *Drosophila melanogaster*, a species in which aggression has been studied primarily in the context of territorial behaviour. In our experiments, males at an attractive food patch were more aggressive towards other males when they were in the presence of their recent mates than when they were in the presence of females mated with other males. Furthermore, while recently mated males accompanied by their mates were more aggressive than virgin males, recently mated males and virgin males showed similar levels of aggression in the presence of females mated with other males. When we allowed focal males to mate inside experimental arenas and then added intruder males, the intruder males spent less time on the food patch, remated with the resident females at lower frequencies and fathered a smaller proportion of offspring when the focals males remained in the arenas than when we removed the focal males. Our results reveal a novel adaptive function of aggression in fruit flies: in addition to fighting to defend attractive food sources that attract prospective mates, males rely on aggression to guard their mates, and such mate guarding enhances their fitness.

Aggression has long been recognized as a primary trait influencing fitness, especially in males, which often fight for territories and prospective mates (Darwin, 1871; Howard, 1920). Fruit flies, *Drosophila melanogaster*, have recently been adopted for a close examination of the genetics and neurobiology of aggression (Alekseyenko et al., 2014; Chen, Lee, Bowens, Huber, & Kravitz, 2002; Dierick & Greenspan, 2006; Zwarts et al., 2011). Because fruit flies are also highly amenable for behavioural, ecological and evolutionary research, we have a unique opportunity for integrating the rapidly accumulating knowledge about the mechanisms that control the varieties of aggressive behaviours with their functional aspects.

Since the first experimental analysis of fruit fly aggression (Dow & Schilcher, 1975), the primary focus in laboratory protocols has been on aggression in the context of territorial behaviour (Certel & Kravitz, 2012; Chen et al., 2002; Dierick & Greenspan, 2006; Hoffmann, 1987b). The limited field work is consistent with the notion that male aggression serves for defending fruits frequented by prospective mates (Markow, 1988). Male aggression, however, can also contribute to other activities such as mate guarding. Male fruit flies defend fruits that are highly suitable for feeding and oviposition. This means that females will most likely remain at their location of mating, because after mating, they increase feeding and then initiate egg laying (Gioti et al., 2012). Remating, however, may be common (Harshman & Clark, 1998) even though recently mated females have lower receptivity than virgin females (Chapman et al., 2003). Because there is a strong last-male sperm precedence in fruit flies (Gromko, Gilbert, & Richmond, 1984; Price, Dyer, & Coyne, 1999), the earlier male to mate will gain little paternity if his recent mate is quick to remate with another male. Thus males can benefit from guarding their mates that remain at the fruit they defend.

Mate guarding has been well studied in many species (Alcock, 1994; Simmons, 2001) and can be expressed in different ways. The most overt way involves cases such as in the dragonfly, *Pachydiplax longipennis*, in which the male remains close to the female after mating and during her oviposition and chases away approaching males (Sherman, 1983). Similarly, in Idaho ground squirrels, *Spermophilus brunneus*, the males stay close to their mates and attack approaching males. Field observations corroborated with genetic tests indeed indicated that males sired the pups

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born to females that they guarded (Sherman, 1989). An alternative form of mate guarding involves the males simply remaining mounted to females after copulation. This probably reduces the guarding males' need for using aggression. For example, male dung flies (*Scatophaga stercoraria*) remain mounted on the females after copulation for the duration of oviposition and thus physically block mounting by other males. Calculations indicate that such mate guarding is advantageous over the alternative strategy of searching for other females (Parker, 1970). In soapberry bugs (*Jadera haematoloma*), copulations can last up to a few days even though sperm transfer takes only a few minutes. The males also remain close to the females during oviposition and remate if other males approach (Carroll, 1991). Another form of mate guarding occurs in a variety of territorial birds. In addition to aggressively defending territories, the males closely follow their mates during their fertile period in order to reduce extrapair copulations (Beasley, 1996; Birkhead, 1979; Chuang-Dobbs, Webster, & Holmes, 2001; Dickinson, 1997; Dickinson & Leonard, 1996). For example, in house wrens, *Troglodytes aedon*, short-term experimental detention of males resulted in higher rates of extrapair copulations and paternity (Brylawski & Whittingham, 2004).

A recent study focusing on the mechanisms of aggression (Yuan, Song, Yang, Jan, & Jan, 2014) hinted at the possibility of mate guarding in fruit flies. Because the natural history of fruit flies described above implies that mate guarding may be beneficial under some realistic field settings, we conducted a set of experiments to critically test the role of aggression in mate guarding. Overall, our goal was to expand the scope of research on aggression in fruit flies in order to place it in a broader ecological perspective. This can help us understand both the mechanisms and fitness consequences of aggression in many animals. Specifically, we predicted (1) that males with their recent mates would be more aggressive than control males, (2) that aggression in the context of mate guarding would decrease female remating frequency with other males and (3) that aggression in the context of mate guarding would increase the paternity of mate guards.

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. 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. We used gentle aspiration to sex and transfer the males into individual food vials, and CO_2 to sex and place females in groups of 20 per food vial. Each 40 ml vial contained 5 ml of the standard fly medium, and the females' vials also contained a dash of live yeast. At the time of testing all flies were 4 days old. We used small amounts of pink fluorescent powder to mark males to allow us to distinguish between males when two males shared an arena. Male colouring was counterbalanced with male treatment.

We conducted all tests in cylindrical arenas (3 cm in diameter, 2.5 cm high) made of Plexiglas. 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). The floor of each arena had a piece of moist filter paper, and each arena contained a circular food patch (1.3 cm in diameter, 1.5 mm high) covered with a live-yeast suspension. We recorded all trials using webcams (Logitech HD Pro C920 and iPad Air). Then, observers blind to fly treatment scored the videos using Noldus software (Noldus Information Technology, Wageningen, The Netherlands). We used generalized linear models (GLMs) when there were independent measures, and we used generalized estimating equations (GEEs) when there were repeated measures (SPSS, IBM Corp., 2011). Unless noted otherwise, the models assumed gamma distributions with log linked functions.

AGGRESSION IN THE CONTEXT OF MATE GUARDING

Methods

We began by examining whether males were more aggressive towards other males in the presence of their recent mates than were control males. We used a protocol modified from Yuan et al. (2014). In the mate-guarding treatment ($N = 30$ arenas), we placed two focal males in an arena with two virgin females and allowed them to mate (Fig. 1a). After both males had finished mating, we waited 10 min and then began video recording the arenas for 30 min. In the nonmates treatment ($N = 30$ arenas), we placed one female and one male in each of two vials and allowed them to mate. Following mating, we discarded the males, placed the two mated females and two virgin focal males in an arena, let them acclimate for 10 min and then began video recording for 30 min (Fig. 1a). From these videos, observers who were blind to the male treatment recorded the total duration of aggression, which included all occurrences of lunging, wing threat, high-level fencing, holding, boxing, tussling and charging (Chen et al., 2002;

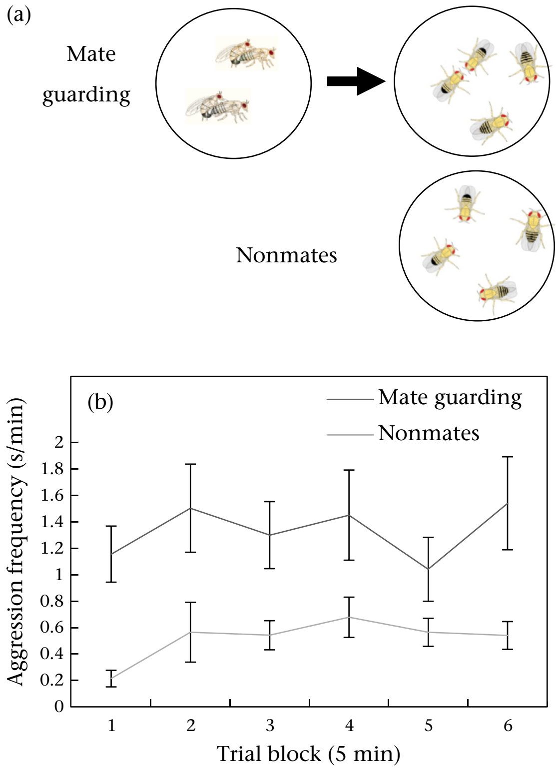

Figure 1. (a) The mate-guarding treatment involved two males in the presence of their recent mates whereas the nonmates treatment had two males together with females recently mated to other males (note that males are distinguished from females by their smaller size and the black tip of their abdomen). (b) Mean ± SE aggression frequency (s/min) per 5 min block per arena in the mate-guarding and nonmates treatments ($N = 60$ arenas).

Dierick & Greenspan, 2006). To assess changes in aggression over time, we separated the 30 min trials into six blocks of 5 min. We predicted higher aggression levels in the mate-guarding condition than in the nonmates condition.

Results

Males were significantly more aggressive towards other males in the mate-guarding treatment than in the nonmates treatment (GEE: Wald $\chi^2_1 = 20.7$, $N = 60$, $P < 0.001$; Fig. 1b). Aggression varied significantly throughout the trials (Wald $\chi^2_5 = 18.3$, $N = 60$, $P < 0.005$), but the interaction between male type and time throughout the trials was not significant (Wald $\chi^2_5 = 8.5$, $N = 60$, $P = 0.13$; Fig. 1b).

AGGRESSION BY MATE GUARDS VERSUS MATED MALES

Methods

The previous experiment indicated that recently mated males in the presence of their mates were more aggressive than focal males in the presence of females recently mated to other males (Fig. 1b). To assess the relative effects of mate guarding and recent mating on male aggression, we compared male aggression under two conditions. In the mate-guarding treatment ($N = 30$), we placed a focal male (guard) and a virgin female in an arena and allowed them to mate. Following mating, we added a virgin intruder male, allowed the flies to acclimate for 10 min and then began video recording for 30 min (Fig. 2a). In the nonmate treatment ($N = 30$), we placed one virgin female and one virgin male in each of two vials and allowed them to mate. Following mating, we discarded the female from one vial and the male from the other vial, and placed the focal male and nonmate female along with a virgin male in the arena, allowed them to acclimate for 10 min and then began video recording for 30 min (Fig. 2b). Observers blind to treatment and male role recorded the duration of aggression performed by each male. We predicted more aggression by the guard males than by the intruder males in the mate-guarding treatment and no difference in aggression levels between the focal males and other males in the nonmate condition. We had to exclude three replicates from the analysis because the male colours were not distinct in two replicates and the video file was corrupted in another replicate.

Intruder males can respond to aggressive male guards by reducing further contact with these males (Figure 1 in Yurkovic, Wang, Basu, & Kravitz, 2006). This means that the duration of aggression may not be the best measure of male behaviour. We thus also quantified the relative dominance of the two males, measured by the time they spent on the food patch. Because our preliminary data indicated that the females spent much of their time on the food patch, we predicted a longer food patch residency by the guard males than by the intruder males in the mate-guarding treatment and no difference in food patch residency between the focal males and other males in the control condition.

Results

The male guards were more aggressive than the intruder males in the mate-guarding treatment (GEE: Wald $\chi^2_1 = 5.3$, $N = 58$, $P < 0.05$; Fig. 2c). Aggression did not vary significantly throughout the trials (Wald $\chi^2_5 = 5.5$, $N = 58$, $P = 0.4$), but the interaction between male type and time throughout the trials was significant (Wald $\chi^2_1 = 20.5$, $N = 58$, $P < 0.001$). In contrast, there was no difference in aggression levels between the focal males and the other males in the nonmate treatment (Wald $\chi^2_1 = 0.8$, $N = 56$, $P = 0.4$; Fig. 2d). Both the levels of aggression throughout the trials (Wald $\chi^2_5 = 19.9$, $N = 56$, $P < 0.01$) and the interaction between male type and time throughout the trials were significant (Wald $\chi^2_5 = 14.1$, $N = 56$, $P < 0.02$).

The food patch residency data paralleled those of male aggression. The male guards spent significantly more time on the food patch than the intruder males (Wald $\chi^2_1 = 5.8$, $N = 58$, $P < 0.02$; Fig. 2e). Patch residency varied significantly throughout the trials (Wald $\chi^2_5 = 34$, $N = 58$, $P < 0.001$), and the interaction between male type and time throughout the trials was significant as well (Wald $\chi^2_5 = 19.5$, $N = 58$, $P < 0.002$). In contrast, there was no difference in food patch residency between the focal males and the other males in the nonmate treatment (Wald $\chi^2_1 = 0.6$, $N = 56$, $P = 0.4$; Fig. 2f). Patch residency varied significantly throughout the trials (Wald $\chi^2_5 = 18.3$, $N = 56$, $P < 0.005$), but the interaction between male type and time throughout the trials was not significant (Wald $\chi^2_5 = 2.3$, $N = 56$, $P = 0.8$). The food patch residency of females was nearly identical in the two treatments (mates present: 33 ± 1.6 s/min; nonmates present: 32.6 ± 1.7 s/min; Wald $\chi^2_1 = 0.01$, $N = 57$, $P = 0.9$) and did not vary throughout the 30 min trials (time throughout the trials: Wald $\chi^2_5 = 2.6$, $N = 57$, $P = 0.8$; treatment∗time interaction: Wald $\chi^2_5 = 3.8$, $N = 57$, $P = 0.6$).

FITNESS BENEFIT OF MATE GUARDING

Methods

The above experiments indicated that mated males showed elevated levels of aggression in the presence of their recent mates and intruder males. Such increased aggression can deter intruder males and thus ensure the mated male's paternity. Here we wished to critically test this possibility. Specifically, we predicted, first, that a female would be less likely to remate with intruder males when guarded by her recent mate than when her recent mate was removed, and, second, that a male remaining to guard his recent mate at a patch would father a higher proportion of his mate's offspring than a male removed from the patch after mating.

We conducted two experiments, the first monitoring only rematings and the second measuring both rematings and paternity. In the first experiment, we used the same wild-derived flies used in all previous experiments. We introduced one male and one female into each arena and allowed them to mate. Following mating, we randomly divided the arenas into two treatments. In the guard-present treatment, we kept the male (guard) and his recent mate and added an intruder into each arena ($N = 30$). In the guard-absent treatment, we removed the mated male and introduced an intruder male into each arena ($N = 30$). Observers blind to arena treatment scanned each arena for matings every 5 min for 4 h. Because matings in *D. melanogaster* last about 15 min (Ashburner, 1989), we could record all matings. As supplementary information, the observers also recorded the locations of each fly (on or off the food patch) and the occurrence of courtship in each arena during each 5 min scan.

In the second experiment, we used a combination of our regular wild-derived flies and wild-derived eye mutants with partially white eyes. We had identified the eye mutation in a few flies descended from our field-collected population and isolated them in a separate population cage maintained under the same conditions described above for the wild-type flies. Our analyses indicated that the eye mutation is autosomal recessive with simple Mendelian inheritance. The protocol was similar to that described above except for the following. We used eye-mutant females, wild-type males as the guards and eye-mutant males as the intruders. After the observation phase had finished, we removed the females from each arena and placed them individually in labelled food vials with

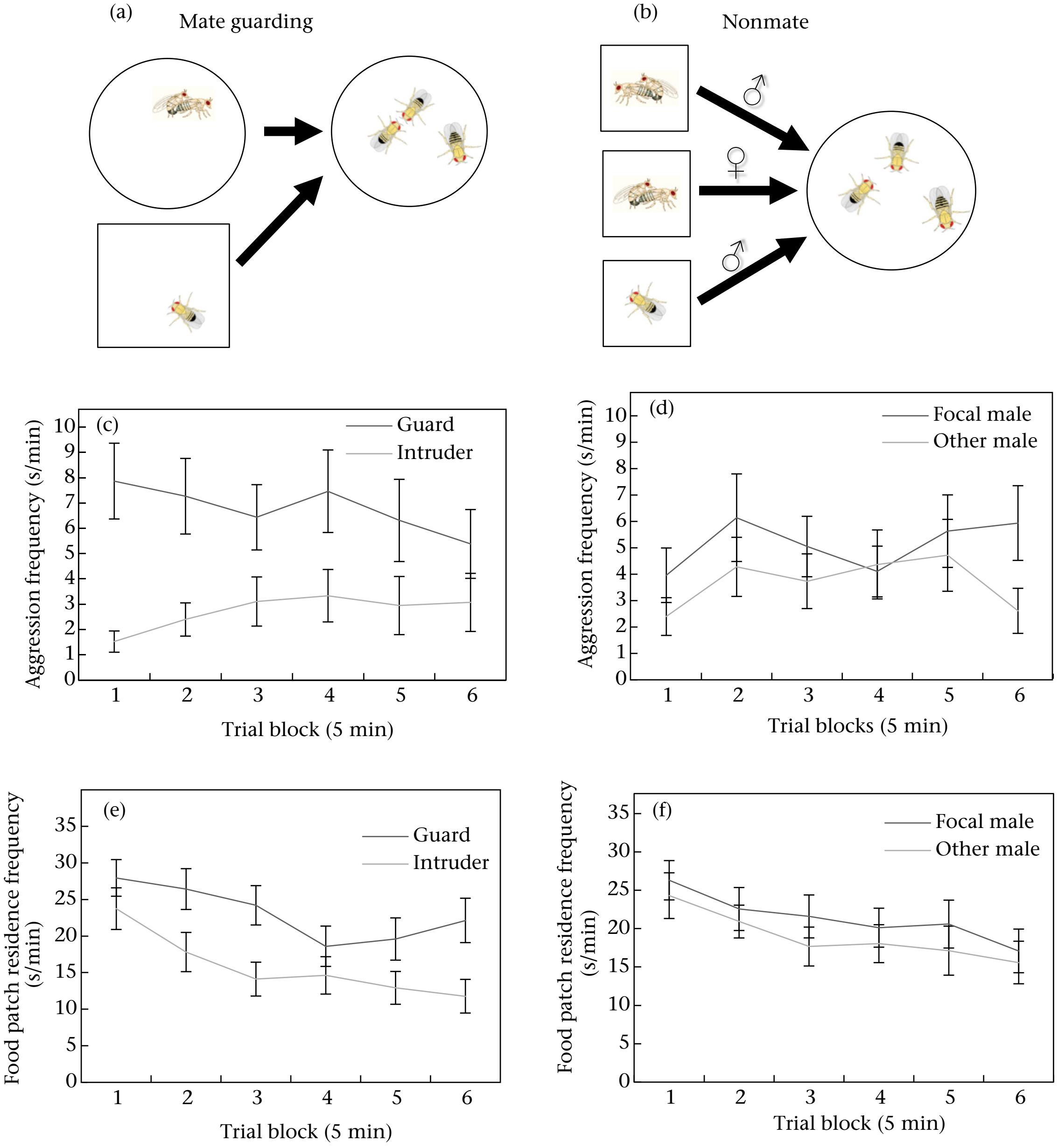

Figure 2. In the mate-guarding treatment (a), we introduced an intruder male into an arena containing a guard male and his recent mate. In the nonmate treatment (b), we placed in an arena a recently mated focal male, a female recently mated to another male, and another male. Panels (c) and (d) depict the mean ± SE aggression frequency (s/min) per 5 min block by each male in the mate-guarding ($N = 58$) and nonmate ($N = 56$) treatments, respectively. Panels (e) and (f) show food patch residency (s/min) per 5 minute block in the mate-guarding ($N = 58$) and nonmate ($N = 56$) treatments, respectively.

a dash of live yeast. We transferred these mated females to fresh vials every other day until they no longer laid fertilized eggs. Observers blind to female treatment counted all wild-type and eye-mutant adult offspring. We analysed the remating data with generalized linear models with multinomial distributions and probit link functions and analysed the progeny data and courtship and location data using generalized linear models with gamma distributions and log link functions.

Results

In the first experiment, females' remating rates with intruders were lower in the presence than in the absence of guard males (GLM: Wald $\chi^2_1 = 3.3$, $N = 60$, $P = 0.07$; Fig. 3a). Intruder males spent significantly less time on the food patch in the presence than in the absence of the guards (Wald $\chi^2_1 = 46.3$, $N = 60$, $P < 0.001$; Fig. 3b) but courted for similar durations in both treatments (Wald $\chi^2_1 = 0.026$, $N = 60$, $P = 0.9$; Fig. 3b).

In the second experiment, females' remating rates with intruder males were also lower in the presence than in the absence of guard males (GLM: Wald $\chi^2_1 = 11.5$, $N = 60$, $P < 0.001$; Fig. 4a). Intruder males spent less time both on the food patch and courting when the guard was present than when he was absent (on food patch: Wald $\chi^2_1 = 35.8$, $N = 60$, $P < 0.001$; courting: Wald $\chi^2_1 = 8.5$, $N = 60$, $P < 0.01$; Fig. 4b). Finally, the guard males fathered a greater proportion of the females' offspring when they were present than when they were absent (Wald $\chi^2_1 = 4.3$, $N = 59$, $P < 0.05$; Fig. 4c).

DISCUSSION

Our major findings were that male fruit flies occupying a food patch with their recent mates were more aggressive towards other

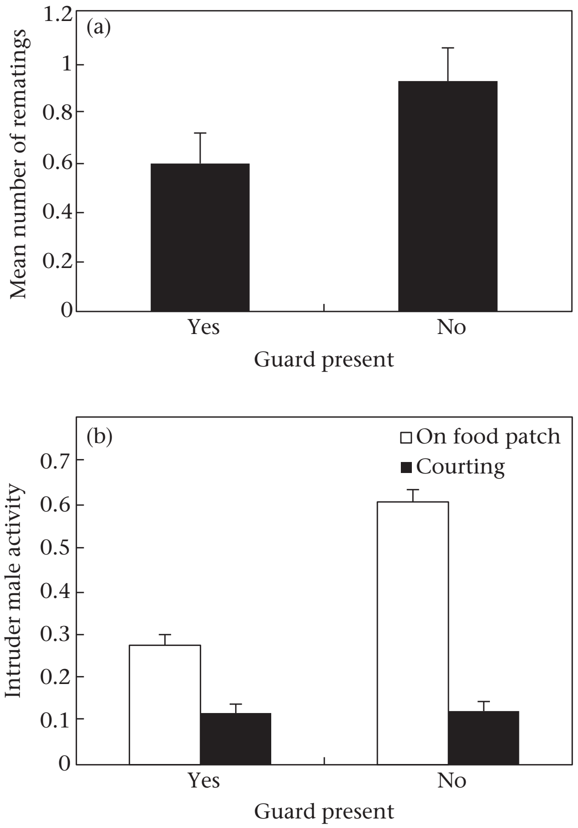

Figure 3. (a) Mean ± SE number of rematings by intruder males when the guard male was either present or absent ($N = 60$ males). (b) Mean ± SE proportion of observations that intruder males were seen on the food patch and courting when the guard male was either present or absent ($N = 60$ males).

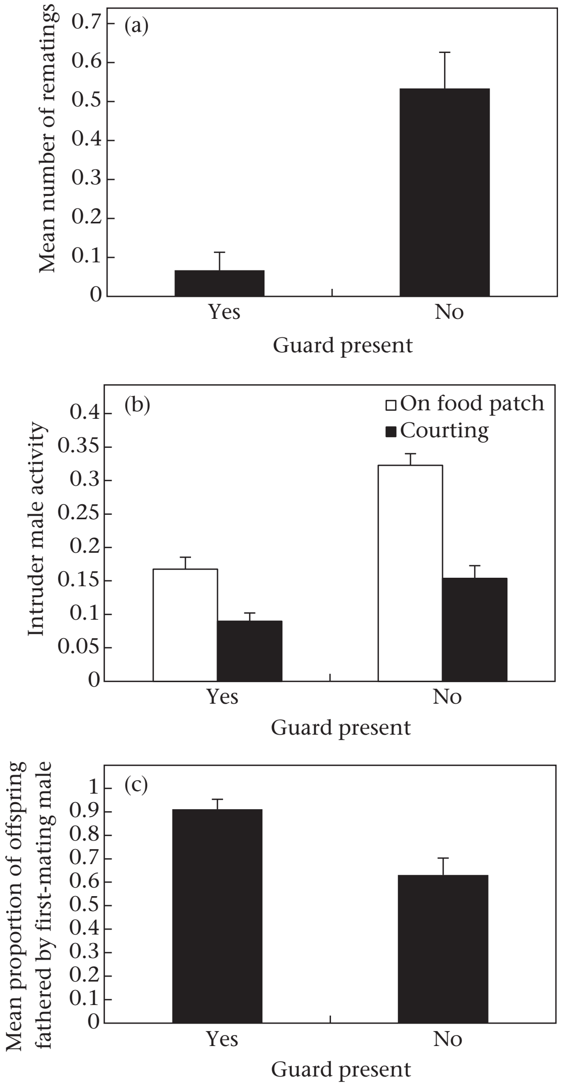

Figure 4. (a) Mean ± SE number of rematings by intruder males when the guard male was either present or absent ($N = 60$ males). (b) Mean ± SE proportion of observations that intruder males were seen on the food patch and courting when the guard male was either present or absent ($N = 60$ males). (c) Mean ± SE proportion of offspring fathered by the guard male when he was either present or absent.

males than were males with nonmate females (Figs. 1 and 2). The removal experiments indicated that such elevated aggression served for mate guarding, because removing the male guards increased the food patch occupancy, remating rates and paternity of intruder males (Figs. 3 and 4). We should note that our experiments do not fully resolve the effects of either mating or experimental transfer into a new resource on male aggression. While mate guarding has been studied in a large variety of species (Alcock, 1994; Simmons, 2001), very few studies have experimentally tested its fitness consequences. Exceptions include a few bird studies in which short-term detentions of males resulted in increased extrapair paternity (Brylawski & Whittingham, 2004; Chuang-Dobbs et al., 2001).

Our experimental results are consistent with the limited information about the natural history of fruit flies (*D. melanogaster*). In settings with dispersed, small, decaying fruits, large males defend the fruits most attractive for feeding and egg laying, which are frequented by females. These males are more likely to mate than are smaller males (Hoffmann & Cacoyianni, 1990; Markow, 1988). The focus of previous behavioural (e.g. Hoffmann, 1987a, 1987b; Hoffmann & Cacoyianni, 1990) and neurogenetic (Chen et al., 2002; Dierick & Greenspan, 2006; Yuan et al., 2014; Zwarts et al., 2011) studies was on male territoriality. From an evolutionary ecological perspective, however, the fruit fly (*D. melanogaster*) mating system is a classical resource defence polygyny (Emlen & Oring, 1977), in which some males monopolize decaying fruit that females require for feeding and egg laying. While the previous research implied that males that monopolize attractive food sources are more likely to acquire mates, our work suggests that an equally or even more important function of male aggression is in reducing the frequency with which a male's recent mates remate with other males.

One can argue that, just by maintaining control of a fruit, the occupying male ensures his paternity. That is, males merely defend their fruit and there is no true mate guarding. Our results, however, indicate that the males show context-dependent aggression, with males at an attractive resource in the presence of their recent mates being more aggressive than males with nonmate females. The best explanation for this context-dependent aggression is that males elevate their levels of aggression in order to deter other males that are attracted to their recent mate. It is indeed likely that, in nature, a fruit occupied by a male and recently mated females attracts more intruder males than a fruit with only a male, because recently mated females emit *cis*-vaccenyl acetate (cVA), which serves as an aggregation pheromone in fruit flies (Bartelt, Schaner, & Jackson, 1985; Wertheim, Allemand, Vet, & Dicke, 2006). Hence an evolved mechanism that generates increased aggression in a resource-defending male fruit fly after mating seems adaptive. In general, mate guarding in male fruit flies is somewhat similar to mate guarding in territorial birds (e.g. Birkhead, 1979; Brylawski & Whittingham, 2004; Dickinson, 1997; Sundberg, 1994). In both systems, males engage in a conspicuous defence of either a resource (fruit flies) or territory (birds) and somewhat less conspicuous protection of their mate.

To further assess the importance of aggression for mate guarding, we will require field data on two key behaviours of recently mated females: (1) their tendency to stay at the fruit where they have just mated and (2) their frequency of remating. Because males fight for the possession of the most desirable fallen fruits, which provide both adult and larval nourishment, we would expect females to frequent these fruits after mating in order to feed and lay eggs. There are currently no field data pertaining to this issue. The other key female behaviour for which we desire field data is the frequency of remating as a function of mating recency. Laboratory data provide a somewhat conflicting picture. On one hand, mating reduces female receptivity, and males find recently mated females much less attractive than virgin females (Chapman et al., 2003; Manning, 1962). Indeed, in fly populations recently established from the wild, short-term tests lasting 15–60 min reveal no rematings in females mated up to 24 h beforehand (Dukas, 2005). On the other hand, at least in small arenas, recently mated females that are incessantly courted by males for a few hours often remate (Billeter, Jagadeesh, Stepek, Azanchi, & Levine, 2012). This was true also in our experiments, in which trials lasting only 30 min had close to zero rematings (data not shown) whereas longer trials lasting 4 h had a high proportion of rematings (Figs 3a, 4a). The most critical field data, which ensured that there were no rematings during trapping, did indicate that females remate (Harshman & Clark, 1998; Ochando, Reyes, & Ayala, 1996), but they did not provide the desired information about the frequency of remating as a function of mating recency.

While our results suggest a novel function of aggression in male fruit flies, we expect that its relative importance will vary depending on the ecological settings, which, in turn, will determine the typically plastic mating system. For example, aggression in the context of resource defence polygyny will be most common in settings with distinct resources and relatively low male density (Emlen & Oring, 1977). Environments with other conditions, including those most common in fruit fly laboratories, which have a single food source and high male density, might select for either other types of aggression or a baseline of low aggression. That is, to achieve our goal of producing a comprehensive synthesis of the types of aggression and their biological bases, we must attend to the subtleties of fruit fly natural history in an evolutionary ecological context in addition to the mechanistic foundations of aggression.

Acknowledgments

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