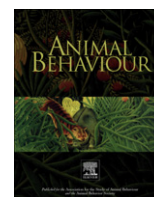




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Costs to females and benefits to males from forced copulations in fruit flies

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Forced copulation, which is perhaps the most extreme form of sexual conflict, is ubiquitous among many species including humans. To better understand the evolution and maintenance of forced copulations, it is imperative to assess their costs and benefits. We followed up on recent studies indicating frequent forced copulations in two wild populations of fruit flies, *Drosophila melanogaster*, and quantified their effects on males and females under controlled laboratory settings. Compared to females that mated consensually, females that were forcibly mated had fewer progeny, higher mortality rates and higher frequencies of wing damage that prevented flight. Males that forcibly copulated fathered much fewer progeny than did males that mated consensually. The reasons for the relatively small reproductive gains to males from forced copulations were the lower female fertility and higher female mortality from forced than consensual copulations as well as the higher frequencies of rematings by females that were forcibly copulated. It is likely that males attempt to force-copulate in spite of the low potential reproductive gain because of the scarcity of sexually receptive females and the consequent low probability of attaining the high fitness associated with consensual matings.

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In most animals, females invest more resources in reproduction than males. This generates divergent mating strategies between the sexes such that males often fight amongst themselves for access to females, females are more selective than males in choosing mates, and males may harm females or coerce them to mate (Darwin 1871; Trivers 1972; Parker 1979; Arnqvist & Rowe 2005). The last strategy constitutes conflict between the sexes, which can lead to an evolutionary arms race where males evolve better armament and females evolve improved defences (Parker 1979; Clutton-Brock & Parker 1995; Arnqvist & Rowe 2005).

There have been excellent research programmes devoted to studying particular types of sexual conflict and their evolutionary consequences (e.g. Arnqvist & Rowe 1995; Rice 1996; Rice et al. 2006; Arnqvist & Tuda 2010; Rowe & Arnqvist 2012). Surprisingly, however, one of the most extreme manifestations of sexual conflict, forced copulation, has received relatively little attention in spite of its prevalence among many animals, including humans (Thornhill 1980; McKinney et al. 1983; Smuts & Smuts 1993; Thornhill & Palmer 2000). Importantly, to understand the evolutionary dynamics resulting from forced copulation, we need to quantify how its exact costs and benefits translate into male and female reproductive success. As far as we know, no study has quantified

reproductive benefits from forced copulations in males. In females, we know of only a single study by Thornhill (1984), in which female scorpion flies (*Panorpa latipennis*) provided with nuptial gifts in consensual matings laid more eggs than females that were forcibly copulated with males offering no nuptial gifts.

Seeley & Dukas (2011) substantiated Markow's (2000) observations of forced copulations in field populations of *Drosophila melanogaster* and *Drosophila simulans* in Arizona by recording forced copulations in a wild Canadian population of *D. melanogaster* as well as in laboratory stocks of Canton-S *D. melanogaster*. Mature males in both populations intensely courted newly eclosed, teneral females, identified by their soft, pale bodies and folded wings. The males attempted to mount these females and succeeded in copulating in about 20% of the trials. The females fought off the males' copulation attempts and continued walking and kicking during copulations. The teneral females had about 10 times longer mating latencies and approximately 25% shorter mating durations compared to sexually mature females, most likely owing to the teneral females' persistent struggles. Limited data indicated that forced copulations resulted in fewer offspring than consensual matings (Seeley & Dukas 2011). Forced copulations also seemed to cause more physical damage and higher mortality rates than consensual matings (C. Seeley & R. Dukas, unpublished data).

The data from forced copulations in fruit flies open up promising opportunities for thorough examinations of the fitness consequences as well as neurogenetic mechanisms involved in this extreme type of sexual conflict. Here, we focus on the former. We conducted a series of experiments to quantify the relative outcomes

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from forced versus consensual matings in both males and females. Specifically, our core predictions regarding females were that, compared to females that mate consensually when mature, females forced to copulate when teneral would have fewer progeny and incur higher frequencies of wing damage and premature death. Our main prediction regarding males was that males that forcibly mate with teneral females would sire fewer progeny than males that consensually mate with mature females.

GENERAL METHODS

We used two lines of fruit flies (*D. melanogaster*) kept under standard conditions in population cages containing a few thousands flies (Seeley & Dukas 2011). The wild type was Canton-S and the marker flies were of the sepia stock, which has been previously used for identifying paternity because the recessive eye-colour mutation does not affect behaviour and fitness (Gromko et al. 1984b; Bretman et al. 2009). We collected teneral females using an aspirator within a few minutes post eclosion. To collect males, we anaesthetized flies with CO₂ 8 h after eclosion and placed males in groups of 20 per regular food vials. We transferred males individually into vials 1 day before each test as this results in higher levels of male sexual activity (R. Dukas, unpublished data). Observers blind to female treatment recorded all the data. The statistical analyses involved ANOVAs on either the raw data when they met ANOVA assumptions or log-transformed numbers, which met ANOVA assumptions after transformations. We used nonparametric tests in cases where transformations failed to normalize the data.

We conducted four experiments, two focusing on the females and two on the males. The two experiments for each sex varied slightly in focus and protocol as detailed below.

FEMALE EXPERIMENT 1

Here we wished to examine costs to females from experiencing male coercion. We used only Canton-S flies in this experiment. Females interacted with either mature females or mature males when teneral and then had the opportunity to mate/remate when sexually mature 2 days later. We had four female treatments that differed in their experience: (1) females that were forcibly mated when teneral on day 1 and did not remate when sexually mature on day 3, (2) females that were forcibly mated when teneral and remated on day 3, (3) females that were placed with males and experienced only courtship and copulation attempts when teneral on day 1 and then mated consensually when sexually mature on day 3, and (4) females that were placed with females when teneral on day 1 and then mated consensually when sexually mature on day 3.

We predicted fewer progeny and higher frequencies of wing damage and premature mortality in females that were forcibly mated when teneral (treatments 1 and 2) than in females that consensually mated when sexually mature (treatments 3 and 4). We further expected that forcibly mated teneral females that did not remate when sexually mature would produce fewer progeny than those that did remate when sexually mature (treatments 1 versus 2, respectively), and that females that mated for the first time when sexually mature would produce fewer progeny when they had been exposed to males rather than females when teneral (treatments 3 versus 4, respectively).

Methods

On day 1, we collected teneral females and placed each in a regular 40 ml vial with two 4-day-old males. We also placed

20 teneral females each in a regular 40 ml vial with two 4-day-old females. We recorded all matings lasting at least 2 min. We set up 168 vials with teneral females and males and recorded matings in 34% of the vials. The average (and range) of forced-mating latency and duration were 30 min (3–71 min) and 698 s (150–1200 s), respectively. Either at the end of mating, or once 2 h had elapsed, we transferred the teneral females into regular food vials with a sprinkle of live yeast and placed them in an environmental chamber. On day 3, we transferred each of the now sexually mature females into a vial containing a 4-day-old male and recorded matings for 1 h. As expected, the frequency of matings of females that were forcibly mated when teneral were much lower than those of the virgin females (28%, $N = 53$, versus 100%, $N = 39$; $\chi^2_1 = 47.6$, $P < 0.001$). Either at the end of mating or once 1 h had elapsed, we transferred the females into regular food vials with a sprinkle of live yeast, recorded their wing damage and placed them in an environmental chamber. We transferred the females into fresh food vials daily and recorded mortality. We kept transferring females into fresh food vials as necessary until they either died or ceased laying fertile eggs, and later counted all adult progeny. Our data set included 96 females.

Wing damage and longevity

Wing damage consisted of part of the wing not extended fully, with a few veins fused together. Flight tests inside cages indicated that females with wing damage had limited or no flight capabilities. Instead, they either walked or hopped. The wing damage analysis included only the 92 females still alive at the time of data recording on day 3. Because most females (91%) survived longer than their sperm stores, we report the proportion of females that died prematurely, defined as death before day 10. We conducted the longevity statistics on the whole data set using Kaplan–Meier survival analyses.

Results

Progeny

The number of progeny varied significantly between female treatments (ANOVA: $F_{3,92} = 7.8$, $P < 0.001$; Fig. 1a). Females that were forcibly mated when teneral had significantly fewer progeny than females consensually mated when sexually mature ($t_{92} = 4$, $P < 0.001$). Note that this difference was not caused by infertility because only 8% and 5% of the females forcibly and consensually mated, respectively, had zero progeny ($\chi^2_1 = 0.3$, $P = 0.7$). Our two additional comparisons, however, revealed no significant differences in the number of progeny between either the females that were forcibly mated when teneral that remated on day 3 and the females that were forcibly mated when teneral that did not remate on day 3 ($t_{92} = 1.15$, $P = 0.25$), or between the females consensually mated when mature that were either exposed to males or females when teneral ($t_{92} = -0.5$, $P = 0.6$; Fig. 1a). An analysis including only the females that lived through the end of the experiment revealed a similar pattern of fewer progeny in females that were forcibly mated when teneral than in females that consensually mated when sexually mature ($t_{83} = 3.4$, $P < 0.001$). That is, the dominant contributor to overall progeny was female fertility rather than premature mortality.

Wing damage and mortality

More forcibly mated females suffered wing damage ($\chi^2_1 = 13.1$, $P < 0.001$) and mortality (Kaplan–Meier: $\chi^2_1 = 3.7$, $P = 0.054$) than females exposed to either males or females when teneral and consensually mated when mature (Fig. 2a). A further analysis comparing the females forcibly mated when teneral versus the females exposed to males but not forcibly mated when teneral

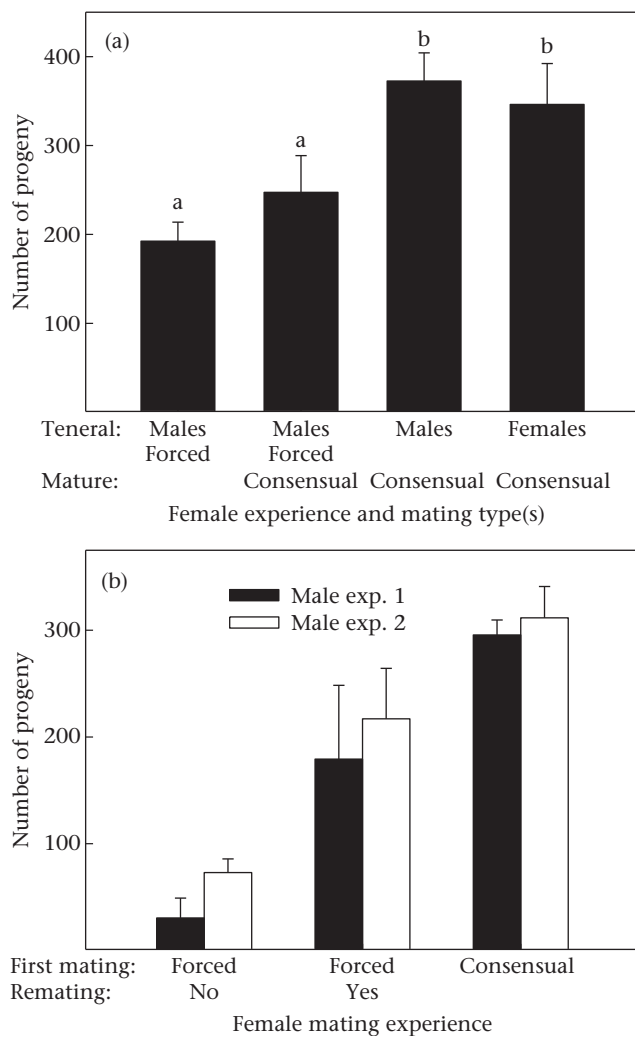


Figure 1. (a) Mean \pm SE number of progeny of female fruit flies ($N = 96$) as a function of their sexual experience in female experiment 1. The four treatments consisted of females that only forcibly mated when teneral, forcibly mated when teneral and remated consensually when mature, exposed to males while teneral and consensually mated when mature, and exposed to females when teneral and mated consensually when mature. (b) Mean \pm SE number of progeny of females that either forcibly mated when teneral or consensually mated when sexually mature in male experiments 1 and 2. All females had the opportunity to remate with other males either 2 or 3 days after the initial mating in male experiment 1 ($N = 34$ females) and 2 ($N = 36$ females), respectively.

indicated that forced matings were associated with significantly higher rates of wing damage ($\chi^2_1 = 5.3$, $P = 0.02$) and higher rates of premature death, but this difference was not significant ($\chi^2_1 = 3.1$, $P = 0.08$; Fig. 2a).

FEMALE EXPERIMENT 2

To further examine the limited data for premature death, we conducted another experiment quantifying female mortality as well as wing damage in the two main treatments of females that were forcibly mated when teneral and females that mated consensually when sexually mature.

Methods

The protocol was similar to female experiment 1 with a few exceptions noted below. On day 1, we collected teneral females,

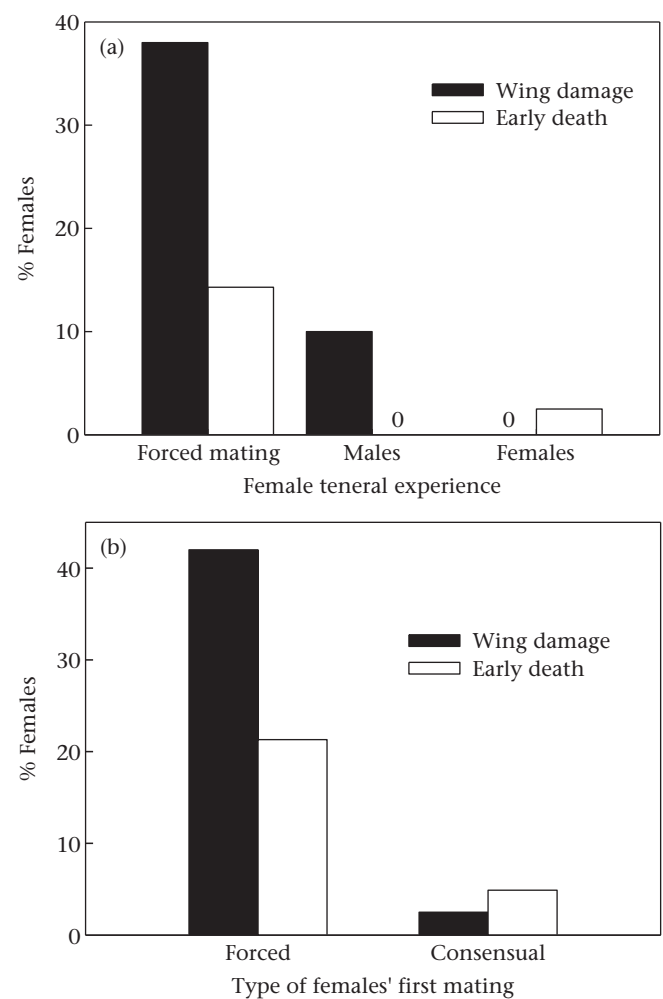


Figure 2. (a) Percentage of female fruit flies that incurred wing damage and early death as a function of their experience while teneral in female experiment 1. Note that no female exposed to males when teneral died prematurely and no female exposed to females when teneral had wing damage. (b) Percentage of females ($N = 76$) that incurred wing damage and early mortality in female experiment 2. Females either were forcibly mated when teneral or had consensual matings when sexually mature.

placed them in food vials and housed them in an environmental chamber. On day 3, we set up mating trials with the sexually mature females collected on day 1 and newly collected teneral females. We had 29% forced matings with the teneral females ($N = 160$) and 100% consensual matings with the sexually mature females ($N = 41$). At the end of matings, we transferred all the mated females individually into food vials and housed them in the environmental chamber. On day 6, we recorded rematings as described above for female experiment 1. Rematings occurred in 53% of the females that were forcibly mated when teneral and in 15% of the females that mated consensually when mature ($\chi^2_1 = 12.3$, $P < 0.001$). We transferred all the females into new food vials and recorded wing damage. We continued transferring females into fresh food vials and recorded mortality through day 10. We tested 88 females.

Results

More females that were forcibly mated when teneral suffered wing damage ($\chi^2_1 = 17.5$, $P < 0.001$) and premature death ($\chi^2_1 = 5.2$, $P = 0.02$) than females that consensually mated when sexually mature (Fig. 2b).

MALE EXPERIMENT 1

Here we wished to quantify the number of progeny that a male gains from forced versus consensual matings under a realistic setting where females have the opportunity to remate.

Methods

The general protocol was similar to the one described above. All females were of the sepia line. Overall, we had two treatments consisting of females that were forcibly mated when teneral and females that consensually mated when sexually mature. In each treatment, half the females mated initially with Canton-S males and had the opportunity to remate with sepia males 2 days later, and the other half mated initially with sepia males and had the opportunity to remate with Canton-S males 2 days later. Specifically, on day 1, we collected teneral females and placed half of them individually inside vials each containing two mature males. Half the vials contained Canton-S males and the other half had sepia males. In addition, we also placed 20 teneral females individually into food vials. When forced matings ended, we transferred the mated females individually into food vials and placed them in an environmental chamber. On day 3, we placed (1) the females that were forcibly mated with Canton-S males on day 1 each inside vials with two sepia males, (2) the females that were forcibly mated with sepia males on day 1 each inside vials with two Canton-S males, (3) 10 virgin females collected on day 1 each inside vials with two Canton-S males, and (4) 10 virgin females collected on day 1 each inside vials with two sepia males. At the end of mating, or after 1 h had elapsed, we moved the females into fresh food vials and placed them in the environmental chamber. Finally, on day 5, we placed (1) the females from treatment 3, which consensually mated with Canton-S males on day 3, each inside vials with two sepia males and (2) the females from treatment 4, which consensually mated with sepia males on day 3, each inside vials with two Canton-S males. We moved all females into fresh vials daily and later counted the eye colours of the eclosing progeny.

Our data set included 15 females that were forcibly mated when teneral and 19 females that mated consensually when sexually mature. Twenty seven per cent of the females that were forcibly mated when teneral and 5% of the females that consensually mated when mature remated 2 days after the first mating ($\chi^2_1 = 3.1$, $P = 0.08$). Five females, all forcibly mated when teneral, experienced early death.

Progeny from forced versus consensual matings

We compared the total number of progeny sired by males that forcibly mated with teneral females versus those of males that mated consensually with sexually mature females. Because the data set remained non-normally distributed after transformation, we present the nonparametric analyses. An ANOVA revealed similar results.

Total female progeny

While the focus in this experiment was on the number of male progeny from forced versus consensual matings, we could also calculate the total number of female progeny from forced and consensual matings. As in female experiment 1, our main prediction was fewer progeny from forced than consensual matings. In addition, we predicted that, among the females forcibly mated when teneral, the females that remated when sexually mature would have more progeny than the females that did not remate. We did not examine the effect of remating on the females initially mated when sexually mature because only a single female remated.

Proportion of progeny from forced and consensual matings

We calculated from the above data the proportion of the females' progeny attributed to the males that engaged in either forced or consensual copulations. Because females that experienced forced matings were more likely to remate than females that mated consensually (Seeley & Dukas 2011), we expected forced copulations to contribute a smaller proportion of the progeny than consensual matings. This analysis had smaller sample sizes because we had to omit females that had zero progeny.

Results

Males that forcibly mated with teneral females sired significantly fewer progeny than males that consensually mated with mature females (Mann–Whitney U test: $U = 274.5$, $N_1 = 15$, $N_2 = 19$, $P < 0.001$; Fig. 3a). Male order had no significant effect ($U = 107$, $N_1 = 15$, $N_2 = 19$, $P = 0.2$). Males that forcibly copulated sired a smaller proportion of the progeny than males that mated consensually ($U = 88.5$, $N_1 = 7$, $N_2 = 19$, $P = 0.06$; Fig. 3b), and forced matings were more likely to be infertile ($\chi^2_1 = 12.1$, $P < 0.001$).

The total number of progeny of females forcibly mated when teneral was significantly smaller than that of females consensually mated when sexually mature (Mann–Whitney U test: $U = 275$, $N_1 = 15$, $N_2 = 19$, $P < 0.001$; Fig. 1b) and females that forcibly mated

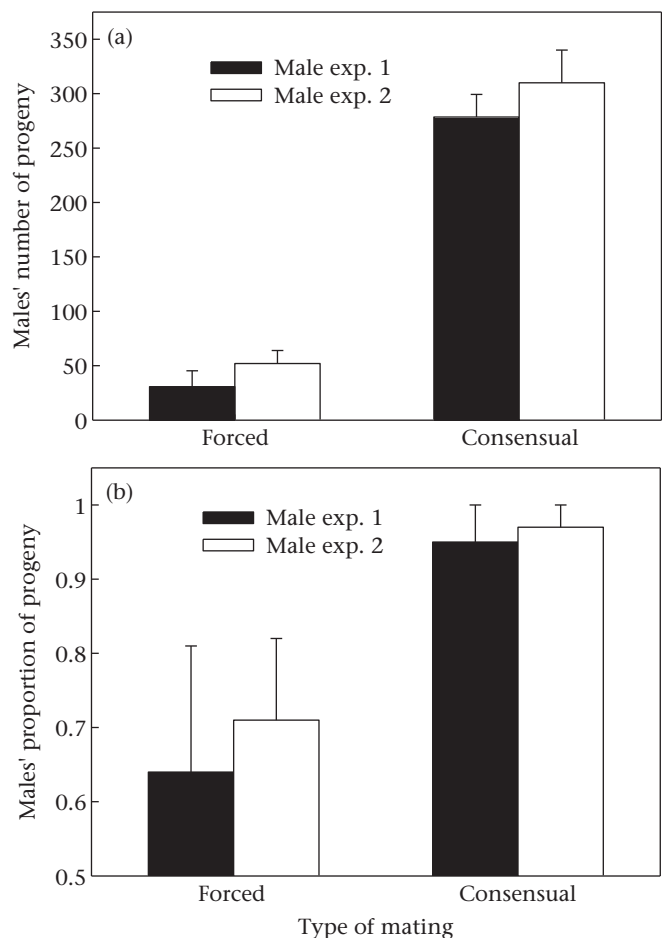


Figure 3. Mean \pm SE (a) number of progeny and (b) proportion of progeny of males that either forcibly mated with teneral females or had consensual matings with mature females. All females had the opportunity to remate with other males either 2 or 3 days after the initial mating in male experiment 1 ($N = 34$ females) and 2 ($N = 36$ females), respectively.

were more likely to have no progeny ($\chi^2_1 = 13.2$, $P < 0.001$). Finally, among the females forcibly mated when teneral, the females that remated when sexually mature had marginally significantly more progeny than the females that did not remate (Mann–Whitney U test: $U = 35.5$, $N_1 = 4$, $N_2 = 11$, $P = 0.056$; Fig. 1b). This pattern, however, disappeared once we eliminated from the analysis females that died within 1 day of remating (Mann–Whitney U test: $U = 19$, $N_1 = 4$, $N_2 = 6$, $P = 0.13$).

MALE EXPERIMENT 2

Methods

This experiment was similar to male experiment 1 except for two modifications. First, we collected one batch of teneral females on day 1 and a second batch on day 3. On day 3, we conducted the mating trials with both teneral and sexually mature females at the same time. The other modification was that we conducted the remating trials at the same time on day 6, 3 rather than 2 days after the initial mating. Our data set included 16 females forcibly mated when teneral and 20 females consensually mated when mature. Thirty one per cent of the females forcibly mated when teneral and 10% of the females mated when mature remated 3 days after the first mating ($\chi^2_1 = 2.6$, $P = 0.1$), and no female experienced early death.

Results

Males that forcibly mated with teneral females sired significantly fewer progeny than males that mated consensually with mature females ($F_{1,32} = 47.3$, $P < 0.001$; Fig. 3a). Male order had no significant effect ($F_{1,32} = 1$, $P = 0.3$). Males that forcibly copulated sired a smaller proportion of the progeny than males that mated consensually ($U = 203.5$, $N_1 = 16$, $N_2 = 20$, $P = 0.03$; Fig. 3b), and forced matings were more likely to be infertile ($\chi^2_1 = 5.6$, $P < 0.02$).

The number of progeny of females forcibly mated when teneral was significantly smaller than that of females consensually mated when sexually mature ($F_{1,34} = 24.4$, $P < 0.001$; Fig. 1b), although all females were fertile. Finally, among the females forcibly mated when teneral, the females that remated when sexually mature had significantly more progeny than the females that did not remate ($F_{1,14} = 15.8$, $P < 0.001$; Fig. 1b).

DISCUSSION

Forced copulation is the most extreme male mating strategy under the ubiquitous setting in which the optimal mating rate is much higher in males than females (Parker 1979; Thornhill 1980; Clutton-Brock & Parker 1995). The obvious female counterstrategy is to have full control over mating. Indeed female fruit flies (*D. melanogaster*) older than 1 h can deflect frequent male advances by decamping, preventing males from attaining a stable mounting position, and by physically preventing male penetration. These female tactics are highly successful as indicated by the fact that, in over a decade of working with a few field-collected and laboratory lines of fruit flies (*D. melanogaster*), we recorded zero matings in sexually immature females 1–20 h old and close to zero rematings in recently mated females under realistic settings of large space, reasonable sex ratio of no more than 2:1 males to females, and trial durations of up to 1 h (e.g. Dukas 2005, 2010; Seeley & Dukas 2011). Recently eclosed, teneral females, however, can neither fly away from males nor fully prevent male mating attempts. Even hiding represents a trade-off because the teneral females' expanding wings must be free from obstruction to attain proper shape before hardening. In spite of the odds against them, the teneral females avoided forced copulations in about 75% of the trials.

Forced copulations were associated with fewer progeny compared to consensual matings (Fig. 1). Mortality within a few days of mating was higher in females that were forcibly copulated than in females that mated consensually (Fig. 2). However, even when we controlled for mortality, female fertility from forced copulations was significantly lower. Finally, because males aggressively pursued teneral females whose wings were not yet extended, forced copulations were associated with wing damage in many of these females whereas sexually mature females that mated consensually had almost no wing damage (Fig. 2). Wing damage prevented females from flying, which, under natural settings, could reduce females' probability of avoiding danger and seeking alternative food sources. That is, in natural settings, wing damage could be a major fitness cost of forced copulations.

Compared to the dramatic cost to females, the benefit to males from forced copulations was rather small. In the two male experiments, males had much fewer offspring from forced than consensual matings (Fig. 3a). There were three main causes for the relatively low male reproductive gain from forced matings. As discussed above, females that were forcibly mated when teneral had lower fertility and higher premature mortality than females that mated consensually when mature. Furthermore, these females were more likely to remate within 2–3 days following the forced mating, meaning that, owing to last male precedence (Gromko et al. 1984a; Pischedda & Rice 2012), males contributed a smaller proportion of the total offspring of forcibly mated females than of consensually mated females (Fig. 3b).

While we could randomly assign newly eclosed females to the no-forced copulation treatment, only about 25% of the teneral females placed with mature males were forcibly mated. Hence, one might argue that the low fecundity of the forcibly mated females merely reflected an association between a females' vulnerability to forced copulation and low fecundity. However, we selected only large and highly mobile teneral females, and the average forced mating latency was over 30 min (Seeley & Dukas 2011). Most likely, variation in male persistence and female age determined the probability of forced mating. Indeed, Seeley & Dukas (2011, their Figure 4) documented a large decrease in the frequency of forced copulation when female age increased from a few minutes to 30 min post eclosion. Nevertheless, because we cannot force forced copulations on females, we cannot reject the hypothetical alternative of some inferior trait associated with both the probability of forced copulation and low fecundity. We should note, however, that our data on wing damage and males' relative reproductive success from forced copulations are not subjected to the above caveat. Wing damage was directly linked to males' harassment of teneral females (Fig. 2a), and the only subgroup of teneral females relevant for quantifying males' reproductive success is the one the males succeed in coercing.

Our data help resolve some puzzles regarding fruit fly behaviour. One such puzzle is that male fruit flies (*D. melanogaster*) intensely court sexually immature females even though such females, between 1 and 20 h old, have odours distinct from those of sexually mature virgin females (Arienti et al. 2010) and always reject the males (e.g. Dukas 2005, 2010; Seeley & Dukas 2011). Second, while the males quickly learn to reduce courtship towards a variety of female classes that reject them, including recently mated and heterospecific females (Dukas 2004, 2005), they persistently court immature females. Third, males also intensely court very young males (McRobert & Tompkins 1983; Curcillo & Tompkins 1987; Dukas 2010), which still do not possess sex-specific cuticular hydrocarbons (Arienti et al. 2010). Finally, experimental elimination of cuticular hydrocarbons made females more attractive to males compared to sexually mature virgin females (Billeter et al. 2009). All these seemingly odd male behaviours can be explained

by the fact that males can gain fitness from forced copulations with very young, sexually immature females. In a setting where the ratio of sexually receptive females to males is close to zero, persistently pursuing young, sexually ambiguous conspecifics may be an optimal male strategy in spite of the little expected fitness gain.

While we have resolved some previously unexplained fruit fly behaviours, our work has also generated a few new questions. First, why did the males in our experiments bother to intensely court the teneral females even though mating could only be achieved through force? One possible answer is that there is a behavioural constraint linking male courtship and mounting attempts. Such constraint is feasible given the strong association between the two when males pursue sexually mature females (see Arnold 1994; Dukas 1998). Another possibility is that teneral females modulate their resistance based on some features of the pursuing males. These features may or may not be identical to the ones used by sexually mature females to assess males. It is thus possible that sexual selection has generated distinct traits that enhance male fruit flies' forced-copulation success. Intriguingly, Markow (2000) reported little to no courtship in males attempting to forcibly mate with teneral females and no signs of resistance by the females, whereas we observed persistent male courtship and obvious struggle by the females in our wild population and in two distinct laboratory populations (Seeley & Dukas 2011; present study). This suggests the existence of geographical variation in male and female tactics involved in forced copulations.

Another question raised by our work is how the females that were forcibly mated when teneral had higher remating propensities than females that consensually mated when mature. This higher remating propensity could be adaptive because, among the females forcibly copulated when teneral, the ones that remated when sexually mature had higher fecundity than the ones that did not remate in two out of three experiments (Fig. 1). It is well known that sperm, seminal fluids and the male-derived pheromone, cis vaccenyl acetate, strongly modify the physiology, behaviour and sexual attractiveness of recently mated females (e.g. Wolfner 2002; Ejima et al. 2007; Fricke et al. 2009; Pitnick et al. 2009; Avila et al. 2010). Perhaps because the forcibly mated teneral females are sexually immature, they are less affected by mating than sexually mature, consensually mated females.

In summary, our results indicate a dramatic cost of forced copulation to females and only a small benefit to males. Although the expected relative male reproductive success from forced versus consensual copulation is low, pursuing forced copulation is likely an optimal male strategy in a setting where the median male reproductive success is probably zero. Our results shed light on a neglected though potentially important feature determining sexual selection in fruit flies, help explain some puzzling aspects of male fruit fly behaviour and lay the foundation for further work quantifying costs, benefits and mechanisms involved in forced copulations and their evolutionary consequences.

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