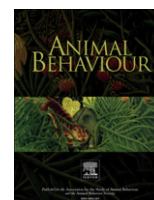




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## Effects of forced copulations on female sexual attractiveness in fruit flies

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Male fruit flies (*Drosophila melanogaster*) force-copulate with females during the females' short vulnerable period following eclosion. Although sexually immature at that time, the females produce viable progeny from forced copulations. While the females could gain fitness from remating with their chosen male when reaching sexual maturity, mating in female fruit flies is typically followed by a long period of reduced attractiveness and receptivity. To test whether forcibly mated females can overcome the typical effects of mating, we quantified the effects of consensual versus forced matings on female attractiveness and receptivity. Forcibly mated females were as attractive to males as same-age virgin females and more attractive than consensually mated females. The forcibly mated females, however, remated at lower frequencies than same-age virgin females but at higher frequencies than consensually mated females. Our results suggest an intriguing outcome of sexual conflict in which males can sire offspring with and reduce the receptivity of forcibly mated females, but these females can retain their attractiveness and partial receptivity to males.

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In most animals, many more males than females seek mates at any given time. One outcome of the consequent sexual conflict between the sexes is that males attempt to forcibly copulate with unwilling females (Parker 1979; Clutton-Brock & Parker 1995; Arnqvist & Rowe 2005). Such forced copulations occur in many species including humans (Thornhill 1980; McKinney et al. 1983; Smuts & Smuts 1993; Thornhill & Palmer 2000). Forced copulations have not been well studied in spite of their potential importance for the evolution of mating systems and sexual selection.

We recently substantiated Markow's (2000) observations of forced copulations in wild fruit flies (*D. melanogaster* and *D. simulans*) in Arizona, U.S.A., by quantifying forced matings in wild as well as laboratory populations of *D. melanogaster*. Male fruit flies vigorously courted newly eclosed females, which ran away from the males, kicked them and attempted to dislodge mounting males. The males, however, succeeded in mating in about 20% of the encounters (Seeley & Dukas 2011). Forcibly mated females had reduced longevity, higher rates of wing damage and fewer progeny than consensually mated females (Dukas & Jongsma 2012). The data on forced copulation in fruit flies open up opportunities for a detailed examination of the evolution of and mechanisms underlying this extreme form of sexual conflict.

In the variety of birds, mammals and insects in which forced copulations have been studied, females mate often and with more than one male (Thornhill 1980; McKinney et al. 1983; Smuts & Smuts 1993; Thornhill & Palmer 2000). In *D. melanogaster* as well as many other insects, however, females typically become unreceptive and unattractive to males for a long period following mating (Gillott 2003). In *D. melanogaster*, these well-studied changes are induced by sperm, seminal fluid and the pheromone, cis-vaccenyl acetate (cVA), which males use to mark females they mate with (Brieger & Butterworth 1970; Gromko et al. 1984; Zawistowski & Richmond 1986; Wolfner 2002; Ejima et al. 2007; Avila et al. 2010). Being unattractive and unreceptive to males may be beneficial to consensually mated females that have chosen their mate because they can then focus on feeding and egg laying. However, perhaps the best strategy available to forcibly mated females is to remate with their chosen male because this may enhance their offspring quality (Partridge 1980; Andersson 1994; Gowaty et al. 2003, 2010). The open question is whether forcibly mated females can counteract the male-induced changes that follow consensual matings. Answering this question can inform us about the evolutionary outcome of the sexual conflict underlying female control of sexual attractiveness and receptivity to males. We thus examined males' courtship of and mating success with forcibly and consensually mated females.

### GENERAL METHODS

We used Canton-S *D. melanogaster* kept under standard conditions in population cages containing a few thousand flies (Seeley &

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Dukas 2011). We collected teneral females using an aspirator within a few minutes posteclosion and collected other flies using CO<sub>2</sub> anaesthesia within 8 h posteclosion. Observers blind to female treatment each watched four vials simultaneously and recorded all the data via a custom-made computer program. For the statistical analyses, we log-transformed the latency data and arcsine square-root transformed the data on proportions of time spent courting. The transformed data met ANOVA assumptions. In the few cases where the latency data remained non-normally distributed after transformations, we used Mann–Whitney *U* tests. We used chi-square tests for the frequency of mating data.

### EXPERIMENT 1: SEXUAL ATTRACTIVENESS OF FORCIBLY MATED VERSUS VIRGIN FEMALES

We started by comparing sexual attractiveness and receptivity of females collected simultaneously when teneral, which were either forcibly mated or not. Based on the well-known effects of mating, we predicted that when 2 days old, females that were forcibly mated when teneral would receive less courtship, have longer mating latencies and a lower frequency of matings than same-age virgin females. Forced matings are shorter than consensual matings (Seeley & Dukas 2011; this study) and it is feasible that their length could influence females' subsequent attractiveness. We thus also quantified the association between females' forced copulation duration and the subsequent courtship they attracted.

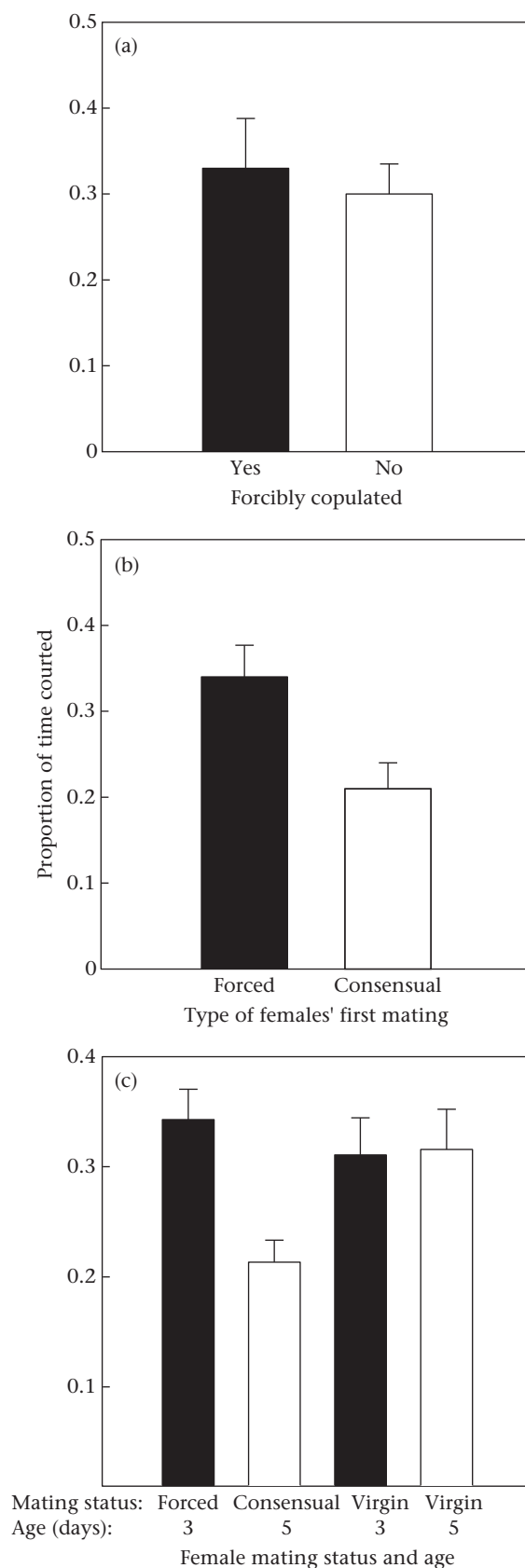
#### Methods

On day 1, we collected virgin teneral females upon eclosion and placed each in a regular 40 ml vial with two virgin 4-day-old males. We recorded all matings lasting at least 2 min. We used this arbitrary threshold to eliminate occasional mountings that did not lead to full copulations. The frequency of forced matings was 29.5%. Either at the end of mating, or once 2 h had elapsed, we transferred females individually into regular food vials containing a sprinkle of live yeast and placed them in an environmental chamber. On day 3, we transferred each female into a vial containing a 4-day-old virgin male. We recorded all courtship behaviour for 15 min in 80 vials, half containing forcibly mated females and half containing virgin females. The time that males spend courting females has often been used to quantify female attractiveness (Siegel & Hall 1979; Ejima et al. 2007), which is not merely a reflection of female receptivity to males, as indicated by the fact that males find sexually immature females (ages 2–20 h), which always reject them, as highly attractive (Dukas & Dukas 2012).

In addition to quantifying female attractiveness, we also wished to measure female mating frequency. Mating latencies, however, are often longer than 15 min. Hence, at the end of the 15 min courtship observation period, we added a second male to each vial with unmated females to allow females some choice. We then continued recording matings for an additional 45 min. The mating latencies thus reflected either mating with the single available male during the first 15 min, or mating with either of the two males over the whole 60 min.

#### Results

Contrary to our prediction, the males spent similar proportions of time courting forcibly mated and virgin females (ANOVA:  $F_{1,77} = 0.3$ ,  $P = 0.6$ ; Fig. 1a). In agreement with our predictions, however, the remating latencies of forcibly mated females were much longer than the mating latencies of the virgin females (mean  $\pm$  SE: 1061  $\pm$  300 s versus 165  $\pm$  22 s;  $F_{1,52} = 24$ ,  $P < 0.001$ ) and the frequency of rematings of forcibly mated females were



**Figure 1.** Mean  $\pm$  SE proportion of time that males spent courting (a) females that either experienced forced mating or had not mated when teneral ( $N = 80$ ), (b) females 3 days after they either were forcibly or consensually mated ( $N = 76$ ) and (c) females that were forcibly or consensually mated, or virgin females of matched ages ( $N = 200$ ).

much lower than those of the virgin females ( $\chi^2_1 = 47.6, P < 0.001$ ; Fig. 2a). Finally, there was no significant association between the mating duration of forcibly mated females on day 1 and the proportion of time males spent courting them on day 3 (linear regression:  $R^2 = 0.02, F_{1,38} = 0.9, P = 0.36$ ).

**EXPERIMENT 2: SEXUAL ATTRACTIVENESS OF FORCIBLY MATED VERSUS CONSENSUALLY MATED FEMALES**

We had predicted that teneral mated females would be relatively unattractive to males compared to same-age virgin females because males typically find recently mated females unattractive (Cook & Cook 1975; Tompkins & Hall 1981; Dukas 2005). The results of experiment 1, however, indicated no difference in males' courtship towards same-age females that either were forcibly mated 2 days earlier or were virgin (Fig. 1a). To clarify this unexpected result, we conducted a follow-up experiment that examined whether males find forcibly mated females more attractive than consensually mated females.

*Methods*

The protocol was similar to experiment 1 with a few exceptions noted below. On day 1, we collected teneral females, placed them in food vials and housed them in an environmental chamber. On day 3, we set up mating trials with newly collected teneral females and the sexually mature females collected on day 1. We had 29% forced matings with the teneral females and 100% consensual matings with the sexually mature females. As expected (Seeley & Dukas 2011; this study, experiment 1), forced matings had longer latencies ( $1930 \pm 148$  s versus  $290 \pm 40$  s, respectively; Mann–Whitney  $U$  test:  $U = 1800.5, N_1 = 47, N_2 = 41, P < 0.001$ ) and shorter durations ( $628 \pm 34.5$  s versus  $845 \pm 24.8$  s, respectively;  $U = 313, N_1 = 47, N_2 = 41, P < 0.001$ ) than consensual matings.

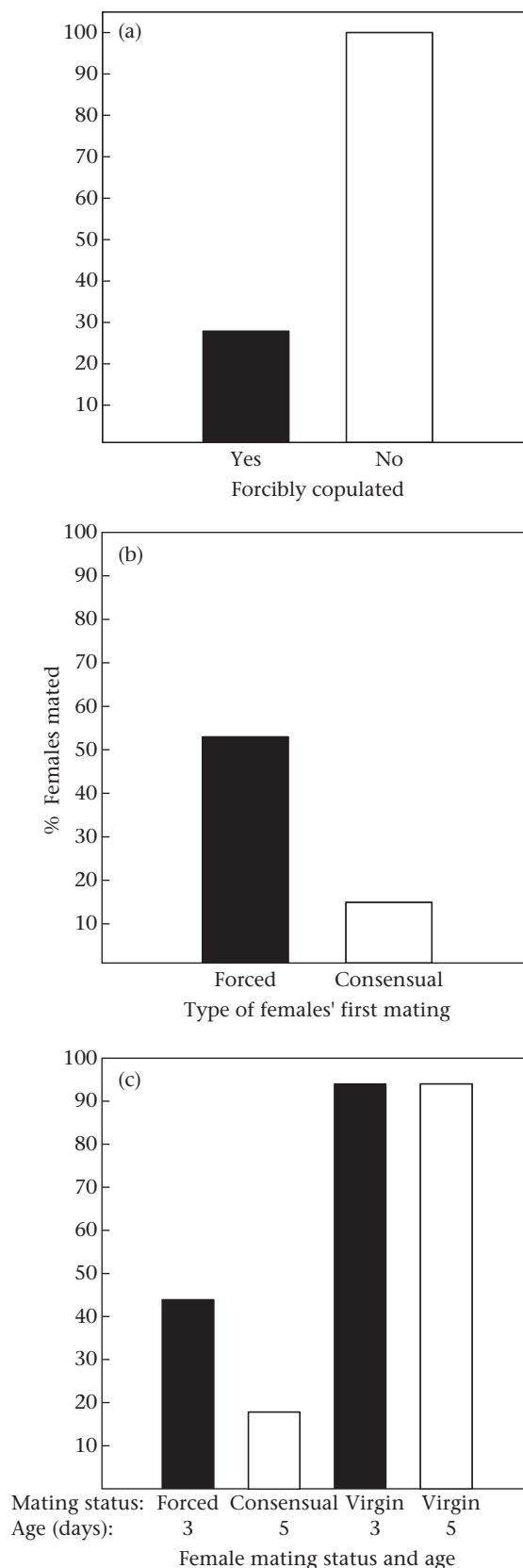
At the end of matings, we transferred all the mated females individually into food vials and housed them in the environmental chamber. We kept these vials for assessing females' fertility from their initial matings. On day 6, we recorded courtship and rematings as described above for experiment 1. We tested 40 consensually mated females and 36 forcibly mated females. The smaller sample size for the latter resulted from a high rate of early mortality.

*Results*

The males spent larger proportions of time courting forcibly mated females than consensually mated females ( $F_{1,74} = 8.2, P < 0.005$ ; Fig. 1b). A further analysis including only the females that were fertile prior to the test showed a similar pattern ( $F_{1,63} = 8.4, P < 0.005$ ). More forcibly mated than consensually mated females remated ( $\chi^2_1 = 12.3, P < 0.001$ ; Fig. 2b). Among the forcibly mated females, fertile and infertile females were equally likely to remate (52% versus 56%, respectively;  $\chi^2_1 = 0.2, P = 0.7$ ). In contrast, among the consensually mated females, fertile females were less likely to remate than infertile females (10.5% versus 100%, respectively;  $\chi^2_1 = 11.9, P < 0.05$ ). Finally, there was no significant association between the mating duration of forcibly mated females on day 3 and the proportion of time males spent courting them on day 6 ( $R^2 = 0.06, F_{1,35} = 2.3, P = 0.14$ ).

**EXPERIMENT 3: CONTROLLING FOR FEMALE AGE**

The comparison of sexual attractiveness of females 3 days after forced or consensual mating implied that consensually mated females were 2 days older than forcibly mated females during the



**Figure 2.** Percentage of females that mated in experiments 1–3 (a–c, respectively). See legend for Fig. 1 for further details.

test. To verify further that the results indicated the effect of forced versus consensual mating rather than age, we repeated the test with the addition of two control treatments consisting of virgin females of ages equal to the forcibly and consensually mated females.

### Methods

The protocol was similar to that of experiment 2 except that we included the two additional treatments of 3-day-old and 5-day-old virgin females. On day 1, we collected teneral females, placed them inside food vials and housed them in an environmental chamber. On day 3, we set up mating trials with newly collected teneral females and half of the sexually mature females collected on day 1. The frequency of forced matings was 42% and the frequency of consensual matings was 93%. Forced matings had longer latencies ( $2031 \pm 123$  s versus  $271 \pm 55$  s, respectively; Mann–Whitney  $U$  test:  $U = 3569$ ,  $N_1 = 71$ ,  $N_2 = 54$ ,  $P < 0.001$ ) and shorter durations ( $700 \pm 29$  s versus  $919 \pm 20$  s, respectively;  $U = 715$ ,  $N_1 = 71$ ,  $N_2 = 60$ ,  $P < 0.001$ ) than consensual matings.

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 courtship and matings for 50 females of each of the four treatments consisting of 3-day-old forcibly mated females, 5-day-old consensually mated females, 3-day-old virgins and 5-day-old virgins.

### Results

The males spent greater proportions of time courting forcibly mated females than consensually mated females ( $F_{1,98} = 13.9$ ,  $P < 0.005$ ; Fig. 1c) A further analysis including only the females that were fertile prior to the test showed a similar pattern ( $F_{1,68} = 11.1$ ,  $P < 0.001$ ). In contrast, the males spent equal proportions of time courting 3-day-old and 5-day-old virgin females ( $F_{1,98} = 0.005$ ,  $P = 0.94$ ; Fig. 1c). More forcibly mated than consensually mated females remated ( $\chi^2_1 = 7.9$ ,  $P < 0.005$ ; Fig. 2c). Among the virgin female treatments, the mating frequency was identical (Fig. 2c). Among the forcibly mated females, fertile and infertile females were equally likely to remate (45.5% versus 43%, respectively;  $\chi^2_1 = 0.03$ ,  $P = 0.85$ ). Among the consensually mated females, fertile females showed a lower nonsignificant tendency to remate than infertile females (14% versus 37.5%, respectively;  $\chi^2_1 = 2.5$ ,  $P = 0.17$ ). Finally, there was no significant association between the mating duration of forcibly mated females on day 3 and the proportion of time males spent courting them on day 6 ( $R^2 = 0.07$ ,  $F_{1,48} = 3.5$ ,  $P = 0.07$ ). The marginally significant  $P$  value was much higher once four outliers consisting of two matings shorter than 5 min and two matings longer than 20 min were excluded ( $R^2 = 0.005$ ,  $F_{1,44} = 0.2$ ,  $P = 0.65$ ).

### DISCUSSION

Our results indicate an intriguing pattern in the outcomes of sexual conflict over mating and subsequent attractiveness and receptivity to remating. Forcibly mated females fully retained their sexual attractiveness to other males (Fig. 1). These females, however, were much slower to remate once courted by males. Remating frequencies of forcibly mated females were lower than mating frequencies of same-age virgin females, but higher than those of consensually mated females (Fig. 2). It is remarkable that males mating with newly eclosed females about 24 h before they reach sexual maturity could cause the strong effects typically associated with consensual matings with sexually mature females, which lay fertile eggs and show low receptivity (Gillott 2003; Avila

et al. 2010). On the other hand, the females retained some control over the outcome of unwanted matings, as indicated by their intact attractiveness and high remating rate 2–3 days after the forced copulation (Fig. 2).

It is likely that our results represent the effect of an evolutionary arms race between males and females, in which males exploit the vulnerability of newly eclosed females and manipulate the females' subsequent behaviour through their sperm and seminal fluid, and females possess counteradaptations that reduce the negative effects they incur from forced copulation. We cannot, however, rule out the alternative that the patterns we observed merely reflect fortuitous outcomes of interactions between sperm and seminal fluid in the reproductive tracts of the forcibly copulated, yet sexually immature, females. Interestingly, and in support of our adaptive interpretation, in the only other experimental analysis of forced copulation, Thornhill (1984) also documented faster return to receptivity by forcibly mated females than consensually mated females in scorpion flies (*Panorpa latipennis*). This could be explained by the fact that, unlike consensual matings, forcibly copulated male scorpion flies did not provide females with nuptial gifts.

While the postmating changes in consensually mated fruit flies have been well studied, they do not help us explain the pattern of differential attractiveness of forcibly and consensually mated females. The sex peptide together with sperm are responsible for a multitude of physiological changes that cause females to increase feeding rate, egg production and egg laying, and to become unreceptive after mating (Chapman et al. 2003; Liu & Kubli 2003). Relatively short-term reductions in female attractiveness are caused by cVA (Jallon 1984), which has a half-life of about 6 h (Bartelt et al. 1985; Schaner et al. 1987). The longer-term reduced female attractiveness in consensually mated females is probably caused by odour changes that have not been well studied (Everaerts et al. 2010).

There were two obvious differences between the forcibly and consensually mated females. First, forcibly mated females had shorter mating durations than consensually mated females. While this may help explain the intact sexual attractiveness of forcibly mated females, we did not find a negative correlation between the duration of forced copulation and subsequent sexual attractiveness measured through male courtship. Furthermore, both seminal fluids and sperm are transferred to females during the first 10 min of mating. Experiments in which matings were interrupted after full sperm transfer resulted in faster female rematings, but the events during and the function of the latter part of matings are not well understood (Gilchrist & Partridge 2000; Chapman & Davies 2004). Finally, it is possible that differential male allocation of seminal fluid proteins to forced versus consensual copulations affected the subsequent distinct female attractiveness (Wigby et al. 2009; Sirot et al. 2011).

The other difference between forcibly and consensually mated females was age. It is possible that teneral females that are forcibly mated retain partial control over the machinery that modulates their postmating physiology and behaviour in a way that allows them to maintain their attractiveness. The mechanisms underlying female postmating behaviour have been well studied (McGraw et al. 2004; Yapici et al. 2008; Hässemeyer et al. 2009; Rezával et al. 2012). Hence, the possible genetic, neurobiological and physiological differences following forced and consensual mating can be examined closely.

Although forced copulations are prevalent, their consequences are not well known. Until now, research on the effects of forced copulations has been limited to observations of physical injuries in a variety of species (McKinney et al. 1983; Smuts & Smuts 1993; Thornhill & Palmer 2000) and a range of psychological effects in



humans (Luo 2000; Thornhill & Palmer 2000; Noll et al. 2003; Mukamana & Brysiewicz 2008). Fruit flies are an excellent model system for experimental analyses of forced copulations and their neurogenetic, behavioural and evolutionary effects.

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