Artificial selection on sexual aggression: Correlated traits and possible trade-offs

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Forced copulation is an extreme form of sexual aggression that can affect the evolution of sex-specific anatomy, morphology, and behavior. To characterize mechanistic and evolutionary aspects of forced copulation, we artificially selected male fruit flies based on their ability to succeed in the naturally prevalent behavior of forced matings with newly eclosed (teneral) females. The low and high forced copulation lineages showed rapid divergence, with the high lineages ultimately showing twice the rates of forced copulation as the low lineages. While males from the high lineages spent more time aggressively pursuing and mounting teneral females, their behavior toward non-teneral and heterospecific females was similar to that of males from the low lineages. Males from the low and high lineages also showed similar levels of male-male aggression. This suggests little or no genetic correlations between sexual aggression and non-aggressive pursuit of females, and between male aggression toward females and males. Surprisingly however, males from the high lineages had twice as high mating success than males from the low lineages when allowed to compete for consensual mating with mature females. In further experiments, we found no evidence for trade-offs associated with high forced mating rates: males from the high lineages did not have lower longevity than males from the low lineages when housed with females, and four generations of relaxed selection did not lead to convergence in forced mating rates. Our data indicate complex interactions among forced copulation success and consensual mating behavior, which we hope to clarify in future genomic work.

KEY WORDS: Aggression, artificial selection, *Drosophila melanogaster*, forced copulation, mate choice, sexual selection.

It has long been recognized that males and females are often in conflict over the optimal mating rates, and that this disparity between the sexes has had large effects on the evolution of sex-specific anatomy, morphology, and behavior (Parker 1979; Arnqvist and Rowe 2005; Chapman 2006). Behaviorally, the most common manifestation of sexual conflict is males' greater motivation for sexual activity and lesser ability to inhibit sexual impulses, as indicated by males' persistent sexual pursuit of reluctant females (Darwin 1871; Thornhill and Palmer 2000; Thompson 2009). In many cases, males' sexual pursuit includes aggression, which can vary in severity from intimidation and harassment to physical injuries (Clutton-Brock and Parker 1995; Muller and Wrangham 2009).

One of the most extreme forms of sexual aggression is forced copulation, where males rely on physical force to insem-inate females that refuse to mate with them. Forced copulation has been reported in a wide variety of species including in-sects, spiders, reptiles, birds, and mammals (Thornhill 1980; McKinney et al. 1983; Smuts and Smuts 1993; Shine et al. 2003; Johns et al. 2009; Muller and Wrangham 2009). Forced copulation is evolutionarily important because it can select for male and female traits that are distinct from those favored by other mechanisms of sexual selection including mate choice, male contests, and post-copulatory processes (Clutton-Brock and Parker 1995; Andersson and Iwasa 1996; Markow 2000). Forced copulation, however, has rarely been subjected to experimental analyses in spite of its prevalence and importance.

To address mechanistic and evolutionary aspects of forced copulation, we focused on a unique system involving newly eclosed, sexually immature female fruit flies (*Drosophila*

melanogaster). For a short period post eclosion, these females, characterized by undeveloped wings and soft cuticle, can neither escape males by flying nor prevent forced intromission. Observations in the field and laboratory indicate that males eagerly pursue such teneral females and succeed in forced copulations in about 20% of their attempts. Although immature, the teneral females store sperm from forced copulations and later produce some offspring (Markow 2000; Seeley and Dukas 2011). While the males gain fitness from forced copulations, the females lose because they incur increased wing damage, fewer progeny and higher mortality rates compared to consensually mated, mature females (Dukas and Jongsma 2012). Immature females older than about 2 h successfully refuse all male advances. They then either reject or accept pursuing males after they reach sexual maturity when approximately 1 day old (Manning 1967; Dukas and Dukas 2012; Dukas and Scott 2015). It is often difficult to distinguish between behaviors associated with male persistence and prospective-mate screening by females, and patterns reflecting male coercion and female resistance (Eberhard 2002; Adler 2010; Baxter et al. 2019). In the fruit fly case, however, we are confident that the copulations with teneral females are forced because they occur during a short window of female vulnerability that is followed by a period in which females successfully reject all males. Consequently, this system allows us to conduct experimental analyses of forced copulations in a leading animal model. This opens up numerous opportunities for advancing our knowledge about the genetics, neurobiology, and evolutionary biology of an extreme form of sexual aggression that occurs in many species.

We have recently documented genetic variation in forced copulation success in male fruit flies (Baxter et al. 2019). To further our investigation of the traits and trade-offs associated with sexual aggression, we artificially selected lineages in which males had either low or high forced copulation success (hereafter low and high lineages respectively). Our goal was to test males from the evolved low and high lineages for several relevant characteristics. First, one would expect that forced copulation success is associated with some form of sexually aggressive behavior, but we could not be certain what this behavior would be. Based on our recent observations (Baxter et al. 2019), we chose to focus on two relevant measures of sexual aggression. We assessed low-level sexual aggression as the duration that males spent pursuing teneral females, and examined high-level sexual aggression as the time males spent attempting to mount females. Second, sexual aggression may be directed specifically toward teneral females or represent a broad male feature expressed toward any female. To characterize the sexually aggressive phenotype, we measured pursuit and mounting by males from the low and high lineages when tested with non-teneral, conspecific females.

Third, it is possible that the feature distinguishing the low and high lineages is not sexual aggression but lack of selectivity. That is, instead of focusing on sexually receptive females, as indicated by their olfactory and gustatory cues (Ferveur 2005; Ejima et al. 2007; Billeter and Levine 2013) and characteristic rejection behaviors (Connolly and Cook 1973; Dukas and Scott 2015), males from the high lineages may be persistent in pursuing and attempting to mate with both receptive and unreceptive females. We tested this possibility by pairing males with mature females of the closely related species, *D. simulans*. Fourth, it is not clear to what degree aggression is context specific (Moyer 1968; Buss and Perry 1992; Wrangham 2018). To address this issue, we tested whether males from the high lineages, which were more sexually aggressive toward teneral females, would also be more aggressive when they can defend an attractive resource against other males (Hoffmann 1987; Chen et al. 2002). This is another type of sexual contest because successful resource defense would allow males to mate with and guard females frequenting the resource for feeding and egg laying (Baxter et al. 2015).

Fifth, while our artificial selection focused on behavior, quantified as either success or failure in forced copulation, the evolved mechanisms underlying that behavior could have been nonbehavioral. Two obvious candidates were body size and sex combs. A variety of studies documented a positive correlation between male-to-female size ratio and male coercion success (Olsson 1995; Crean and Gilburn 1998; Perry and Rowe 2011). Hence, we tested whether males from the high lineages were larger than males from the low lineages. Male fruit flies possess an array of bristles resembling a comb on their front legs, which they use to secure their holding on top of females during copulation. These sex combs enhance their mating success (Cook 1977; Ng and Kopp 2008; Hurtado-Gonzales et al. 2015). Similar sex-specific structures that males use for grasping females prior to and during copulation are known in other species (Darwin 1871; Arnqvist and Rowe 2005). We thus tested whether males from the high lineages evolved more elaborate sex combs. Sixth, we were interested in quantifying how selection on forced copulation success would affect males' mating success with mature females, which can exert choice. Do sexually aggressive males possess traits that females find unattractive? A trade-off between forced and consensual mating success could explain the relatively low rates of forced copulation success in flies derived from the wild. Intriguingly, two independent studies involving artificial selection on male-male aggression reported lower mating success in the aggressive than control lineages (Dierick and Greenspan 2006; Penn et al. 2010). Hence we tested mating success of males from the low and high lineages under no choice (1 male + 1 female) and choice (1 male from each lineage + 1 female) trials.

Seventh, because we assumed that natural selection maintains relatively low levels of forced copulation, we wished to

assess trade-offs associated with high rates of forced copulation success. We expected males from the high lineages to spend more time pursuing females (Baxter et al. 2019) and anticipated that this activity could increase their mortality rates (Cordts and Partridge 1996). Thus, we tested the longevity of males from the low and high lineages while housed with females. Finally, as another test for possible trade-offs associated with forced copulation rates, we simultaneously compared forced copulation rates in the evolved lineages and in lineages subjected to four generations of relaxed selection. If forced copulation rates that are lower or higher than the natural levels have some unknown fitness costs, then we would expect relaxed selection to lead to increased forced copulation rates in the low lineages and reduced forced copulation rates in the high lineages.

To summarize, we first predicted that artificial selection on forced copulation rates would generate divergent lineages of low and high forced copulation success. In tests conducted on the evolved, divergent lineages, we predicted that, compared to males from the low forced copulation lineages, males from the high forced copulation lineages would: (i) be more sexually aggressive toward teneral females as indicated by greater durations of pursuit and mounting attempts; (ii) be more sexually aggressive and persistent in pursuing and mounting non-teneral, conspecific females; (iii) be less selective as indicated by greater durations of pursuit and mounting attempts directed towards females of the closely related species, *D. simulans*; (iv) be more aggressive toward males; (v) have larger body sizes and more bristles in their sex combs; (vi) have lower mating success with young, mature females in no choice (1 male and 1 female) and choice (1 male from each lineage + 1 female) trials; and (vii) have shorter lifespan when housed with females. Finally, we predicted that the evolved lineages belonging to both low and high forced copulation success would incur unspecified fitness costs, which we could infer if they converged toward less extreme forced copulation rates under relaxed selection.

Methods

ARTIFICIAL SELECTION

We collected several hundred female fruit flies in various locations in and around Hamilton, Ontario in summer 2018 and placed them individually in food vials each containing 5 mL of standard medium (1 L standard food contained water, 90 g sucrose, 75 g cornmeal, 10 g agar, 32 g yeast, and 2 g methyl paraben dissolved in 20 mL ethanol) and sprinkled with ≈10 mg live yeast. We inspected male offspring, discarded the vials containing *D. simulans*, and retained 500 vials containing *D. melanogaster*. We mixed the F1 flies, placed them in six population cages measuring 20 × 20 × 30 cm, allowed them to lay eggs, and initiated testing of the F2 flies.

Owing to the large number of flies being tested, we conducted trials over 3 days. For each of the 3 test days, we sexed 280 males and 180 females within 8 h of eclosion under light CO_2 anesthesia. We placed males in individual food vials, and females in groups of 10 in food vials with a sprinkle of live yeast. We tested the males when they were 3 days old by aspirating a newly eclosed teneral female from the baseline population into each male's holding vial. To increase fly encounter rates within vials, we lowered the foam plug in each vial to reduce the space above the food to a height of 1.5 cm, generating a volume of about 10 mL. We observed the vials for 2 h and recorded all forced copulations. We then randomly collected 48 males that did not forcibly copulate to initiate a low forced copulation lineage (Fig. 1A), and randomly collected 48 males that forcibly copulated to initiate a high forced copulation lineage (Fig. 1B). For each lineage, we placed a group of four males with four mature, virgin, 3 day old females in each of 12 food vials sprinkled with live yeast and let the females lay eggs. We transferred the flies into new food vials twice more, so we had three sets of 12 vials for each lineage. By repeating this protocol over 3 days, we generated three low forced copulation lineages and three high forced copulation lineages.

After the initial setup of the six lineages, we started a 2-week cycle in which we placed all vials in an environment chamber at 27°C and 50% RH, with a 12:12 light:dark cycle. About 10 days after the last egg laying, we collected newly eclosed flies. We sexed 80 males per low forced copulation lineage, 250 males per high forced copulation lineage, and 80 females per lineage. We had to test a larger number of males from the high than low lineages in order to ensure that we had 48 males for the next generation while assuming ≈20% forced copulation rates. We kept and tested males as detailed above. We tested one low and one high lineage per day over 3 days and let observers record each forced copulation. Throughout the study, the teneral females always came from the large baseline population, which we kept in two population cages subsequent to the initial setup. After the test, we discarded the teneral females, randomly collected 48 males that did not force copulate from each low lineages, and 48 males that forced copulated from each high lineage. We mated these males with mature, virgin females from their own lineage. That is, we conducted selection on the males only. We performed 20 generations of selection during which we tested 15,960 males from the three low and three high lineages.

EXPERIMENTS ON THE EVOLVED LINEAGES

With one exception, we conducted the experiments on flies that were subjected to two generations of relaxed selection in order to eliminate parental effects. Flies had been subjected to 15–20 generations of artificial selection prior to the two generations of relaxed selection. The exception was the experiment assessing the effect of relaxed selection. Here flies experienced four
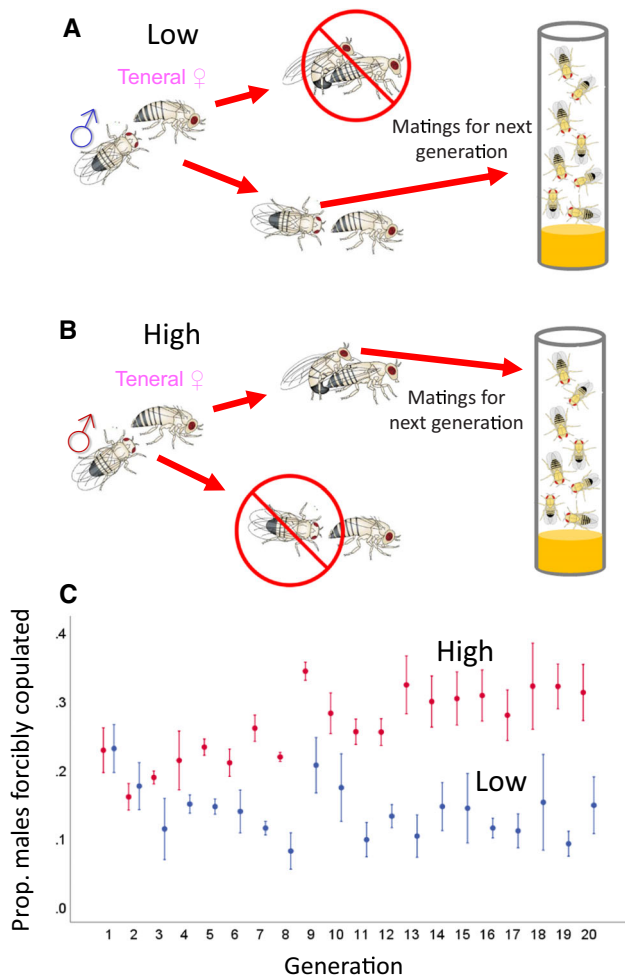
Figure 1. The artificial selection protocol involved selecting 48 males that failed to forcibly copulate with teneral females from each of the 3 low forced copulation lineages (A), and selecting 48 males that forcibly copulated with teneral females from each of the 3 high forced copulation lineages (B). The selected males then mated with mature females from their own lineages to father the next generation. (C) The proportion (±1 SE) of males from the low (blue) and high (red) lineages that forcibly copulated with teneral females over 20 generations of selection. The values depicted are the means and SEs of the 3 low and 3 high lineages for each generation. The full data set included 15,960 males.

generations of relaxed selection prior to the test. In all the experiments, males were 3 days old and females were from our baseline population as described above. As with the artificial selection protocol, we staggered fly eclosion over 3 days in order to test males from one low and one high lineage each day. Observers blind to males' treatment identity conducted all the data recording.

Pursuit and mounting of teneral females

Here, we tested the prediction that males from the high lineages would spend longer durations pursuing and mounting teneral females than would males from the low lineages. We collected and stored flies as described above. We then added one teneral female into each of two male vials and lowered the foam plug in each vial to reduce the space above the food to a height of 1.5 cm as in the artificial selection trials. An observer recorded for 15 min all pursuit and mounting attempts using an iPod touch sixth generation equipped with the Drosophila Assay Assistant app. We defined pursuit as close following of the teneral female, and mounting as any attempt to move into copulation position, which involves the male curling his abdomen while climbing on the female. Our total sample size was 96 trials. During the observations, forced matings occurred in 12 trials (5 with low and 7 with high forced copulation males). In these trials, we stopped observations at the onset of mating and weighted the pursuit and mounting data to compensate for the shorter times available.

Pursuit and mounting of non-teneral females

Here, we tested the prediction that males from the high lineages would be more sexually aggressive and persistent in pursuing and mounting non-teneral, conspecific females than would males from the low lineages. To assess persistence, we recorded male behavior during the first 15 min of a trial, and resumed recording 30 min later during minutes 45–60. Here, we conducted video recordings, which required different types of arenas because it is difficult to score fly behavior from video recordings of vials. The arenas were similar to the ones we have used successfully in previous research (Baxter et al. 2019; Filice and Dukas 2019) and consisted of polystyrene Petri dishes that were 35mm in diameter and 8 mm high, with a volume of about 8.5 mL. We applied a slippery coating of Surfasil (Thermo Fisher, Ottawa, ON, Canada) to the walls and ceilings of the arenas in order to restrict flies to the floors of the arenas. We covered the bottom of each arena with filter paper and placed at their center a circular food patch made of standard medium and measuring 13 mm in diameter and 1.5 mm high. At the middle of the food patch, we dispensed about 25 mg of a thick suspension made of 5 g live yeast in 10 mL grapefruit juice to create a ball 3 mm in diameter. This combination is highly attractive to both male and female fruit flies and effective at eliciting courtship and aggression. We aspirated flies through a 3 mm hole in the ceiling, which we covered with clear tape afterward.

Each trial, we introduced into each arena a single male from either the low or high lineage and a non-teneral female younger than 16 h. Such immature, non-teneral females are highly attractive to males but always reject them, so this is an effective way for standardizing female response to males (Dukas and Scott 2015). We then immediately began video recording using Logitech HD Pro c920 webcams. We recorded minutes 0–15 and 45–60. Later, observers scored the videos using BORIS observation software (Friard and Gamba 2016). For each arena, an observer recorded the durations that each male pursued and

mounted the female. We omitted five trials from the analysis, two (1 low and 1 high) due to matings, one (low) owing to female escape, and two (low) in which males spent over 50% of the trials on the ceiling. Hence, our final data set contained 85 trials.

Pursuit and mounting of heterospecific females

Here, we tested the prediction that, compared to males from the low lineages, males from the high lineages would be less selective in their courtship in general, which could be indicated by their greater durations of pursuit and mounting attempts directed toward females of the closely related species, *D. simulans*. The protocol was identical to the one just described for non-teneral conspecific females except that the females here were 4-day old female *D. simulans* from a population collected locally in summer 2018. We omitted eight trials from the analysis, five (3 low and 2 high) due to matings, and three (1 low and 2 high) in which males spent over 50% of the trials on the ceiling. Hence, our final data set contained 82 trials.

Male-male aggression

To assess possible genetic correlation between types of aggression, we tested whether males from the high lineages would be more aggressive toward males than would males from the low lineages. We used arenas identical to the ones just described. We placed in each arena two males from the same lineage and video recorded for 15 min. Later observers scored the videos using BORIS and recorded the durations of all aggressive acts including wing threat, lunging, holding, boxing, and tussling. We report total aggression duration, which is highly positively correlated with lunging, the most common aggressive act (Guo and Dukas 2020). We omitted a single trial (low) in which one male spent over 50% of the trial on the ceiling. Hence, our final sample size included 89 trials.

Size and sex combs

Here, we tested the prediction that males from the high lineages would have larger body sizes and more bristles in their sex combs than would males from the low lineages. To quantify body size, we measured wing length as the linear distance between the humeral-costal break and the end of the third longitudinal vein (Gilchrist and Partridge 1999). We mounted each right wing onto a microscope slide, photographed the wing using a dissecting microscope equipped with a digital camera, and later measured its length using ImageJ (Schneider et al. 2012). We collected wings of 30 males from each of the six low and high lineages, but could not measure 12 wings owing to damage. Hence our final sample size was 168 wings. We also collected the right legs of 30 males from each of the six low and high lineages and counted the number of bristles using a dissecting microscope. Our total sample size was 180 males.

Mating success with mature females

No choice: Here, we tested whether mature females would be more likely to reject males from the high than low lineages. This experiment involved introducing a 2-day old, virgin female into each male vial and recording all matings that occurred within 1 h. We tested 50 males from each lineage for a total sample size of 300 males.

Choice: In this experiment, we again tested for males' mating success with mature females. Here, however, we allowed females to choose between males from the low and high lineages. Such apparent choice, however, could be determined by competitive interactions between males (Baxter et al. 2018). For each trial, we introduced into a regular vial a 2-day old, virgin female, one male from a low lineage and one male from a high lineage and recorded all matings that occurred within 1 h. To distinguish between the males, half the trials included low males dusted with pink fluorescent powder and the other half had high males with the pink powder. We tested 100 males from each lineage. We set up 300 trials each consisting of pairs of low + high males and observed 200 matings.

Longevity

Here, we tested for a possible trade-off associated with high forced copulation success. We predicted that, when housed with females, males from the high lineages would have shorter lifespan than would males from the low lineages. The protocol included two treatments, males held individually and males held with two females. Each treatment had 120 replicates consisting of 20 males from each of the three low and three high lineages. During the experiment, we lost three males so our final data set included 237 males. We initiated the experiment by aspirating into each vial a 3-day old male. We then added to each vial of the female treatment 2 immature females. Subsequently, we checked all vials every day and noted male mortality. We replaced dead females with new ones. We moved flies twice a week into fresh food vials and replaced all females every 2 weeks.

Effects of relaxed selection

As another test for possible trade-offs associated with low or high forced copulation success, we compared the forced copulation success of lineages after 19 generations of artificial selection to that of lineages subjected to four generations of relaxed selection. We predicted that the evolved lineages belonging to both low and high forced copulation success would incur unspecified fitness costs, which we could infer if they converged toward less extreme forced copulation rates under relaxed selection. To this end, we collected flies from each of the three low and three high forced copulation lineages in generation 16 and maintained them under similar numbers and conditions to

those of the artificial selection lineages for four generations with no selection (generations 16–19). We then tested males from the six relaxed selection and six artificial selection lineages in generation 20. On each of 3 successive days, we tested 60 males from each of four lineages: low and high artificially selected lineages, and the relaxed selection lineages derived from those lineages at generation 16. Our protocol was identical to the one used during artificial selection. Our total sample size was 720 males.

Statistics

We analyzed the generalized linear mixed-effects models (GLMMs) in R version 3.6.2 (R Core Team 2019) with the package lme4 version 1.1-21 (Bates et al. 2015). We report Wald χ^2 values generated with the Anova function from the car package version 3.0-6 (Fox and Weisberg 2019). We analyzed the linear mixed-effects models (LMMs) in SPSS version 26 (IBM 2019). With one exception noted below, all models included treatment (low or high) as a fixed factor, and lineage nested within treatment as a random factor. We verified model fits by visually inspecting plots of model residuals. We modeled the artificial selection data with a GLMM with binomial distribution. This model also included generation and treatment by generation interaction as fixed factors. Using the testOverdispersion function in the DHARMa package (Hartig 2017), we found no evidence of overdispersion in the data set ($P = 0.92$). In addition, we modeled the artificial selection data with a linear model (LM). In this model, the dependent factor was the arcsine square root proportion of forced copulations for each lineage and the independent fixed factors were treatment, generation, and treatment by generation interaction.

We log-transformed the pursuit and mounting data for the teneral, non-teneral, and heterospecific females and analyzed the data with LMMs. The non-teneral and heterospecific models also included time period (0–15 and 45–60 min) and treatment by time-period interaction as fixed factors. We used an LMM on log-transformed data for the analysis of male-male aggression and LMMs for the wing length and sex comb data. We used GLMMs with binomial distribution for the binary mating success data. The mate choice model also included male color marking (blank or pink) as a fixed factor and arena as a random factor. We log transformed the longevity data and also included in the LMM female presence as a fixed factor. Finally, we modeled the forced copulation data from the effects of relaxed selection experiment with a GLMM with binomial distribution. The model also included the selection type (artificial or relaxed) and selection type by treatment (low or high) interaction as fixed factors. All the data sets are available on Dryad (Dukas 2020).

Results
ARTIFICIAL SELECTION

The low and high lineages showed rapid divergence, with the high lineages having twice as much forced copulation success after 20 generations (treatment by generation interaction (i) from the GLMM: Wald $\chi^2_1 = 60.8$, $P < 0.001$; (ii) from the LM: $F_{19,80} = 1.8$, $P < 0.05$; Fig. 1C; Fig. S1 and Table S1).

EXPERIMENTS ON THE EVOLVED LINEAGES
#### Pursuit and mounting of teneral females
Males from the high forced copulation lineages spent more time pursuing and mounting teneral females than did males from the low forced copulation lineages (LMM, pursuit: $F_{1,4} = 5.4$, $P = 0.08$; mounting: $F_{1,4} = 31.9$, $P < 0.05$; Fig. 2).

Pursuit and mounting of non-teneral females
Males from the low and high lineages showed no difference in the duration they spent pursuing and mounting non-teneral females
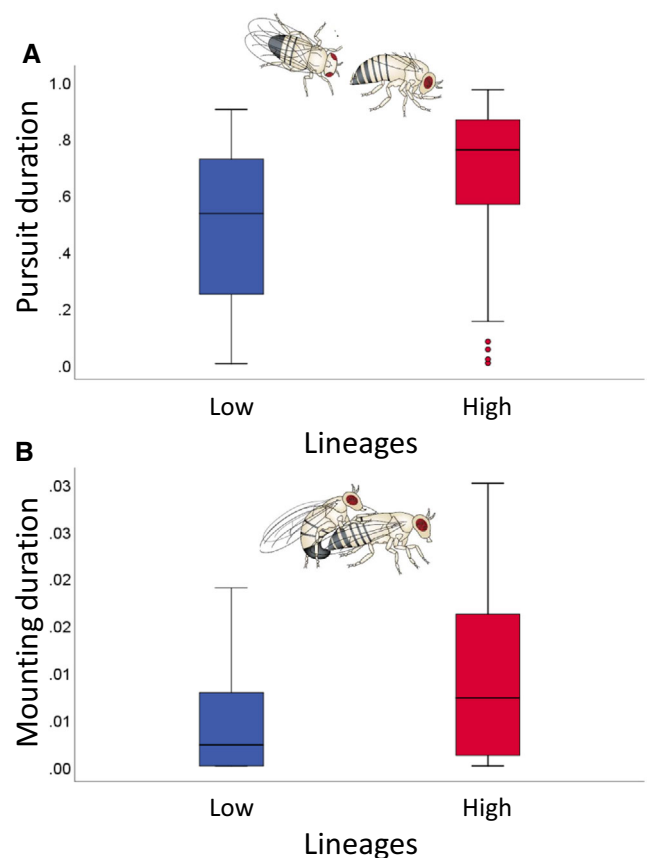
Figure 2. The proportion of time males from the low and high lineages spent pursuing and mounting teneral females. The horizontal lines in the boxplots show the medians, the boxes contain the middle 50% of data (interquartile range, IQR), the whiskers above and below each box represent values within ±1.5 of the IQR, and points depict outliers. *N* = 96 trials.

A

B
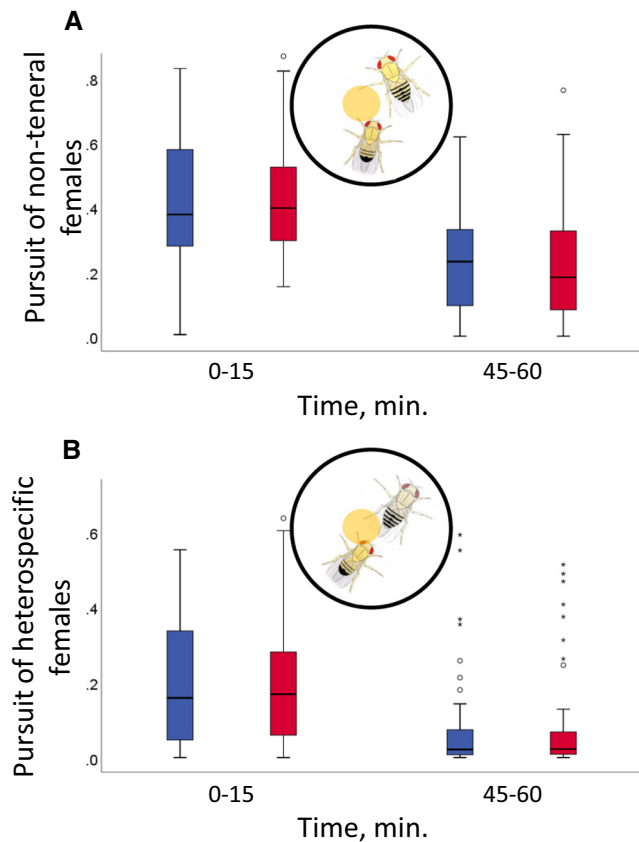
Figure 3. The proportion of time males from the low (blue) and high (red) lineages spent pursuing (A) non-teneral conspecific females (*N* = 85 trials), and (B) heterospecific, *D. simulans* females (*N* = 82 trials). Data are from the first 15 min and minutes 45–60 of each trial.

(LMM, pursuit: $F_{1,4} = 0.02$, $P = 0.9$; mounting: $F_{1,4} = 0.1$, $P = 0.75$; Fig. 3A; Fig. S2A). While males of both treatments showed a large decline in pursuit and mounting over 1 h, the magnitude of decline was similar in the low and high treatments (pursuit: time-period effect, $F_{1,162} = 31.2$, $P < 0.001$; treatment by time-period interaction, $F_{1,162} = 1.2$, $P = 0.28$. Mounting: time-period effect, $F_{1,162} = 12.4$, $P < 0.001$; treatment by time-period interaction: $F_{1,162} = 0.18$, $P = 0.7$; Fig. 3A; Fig. S2A).

Pursuit and mounting of heterospecific females

There were no differences between the males of the low and high lineages in the durations they spent pursuing and mounting heterospecific females (LMM, pursuit: $F_{1,4} = 0.002$, $P = 0.97$; mounting: $F_{1,4} = 0.1$, $P = 0.76$; Fig. 3B; Fig. S2B). Males from both treatments showed a similar reduction in pursuit and mounting over time (pursuit: session effect, $F_{1,156} = 26.2$, $P < 0.001$; treatment by session interaction: $F_{1,156} = 0.9$, $P = 0.77$; Mounting: session effect, $F_{1,156} = 19.9$, $P < 0.001$; treatment by session interaction, $F_{1,156} = 0.33$, $P = 0.23$, Fig. 3B; Fig. S2B).
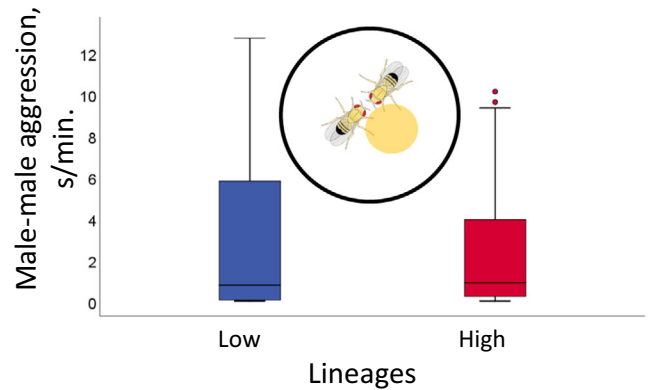
Figure 4. The duration of male-male aggressive activities by males of the low and high lineages (*N* = 89 trials).

Male-male aggression

Males of the low and high forced copulation lineages showed similar levels of male-male aggression ($F_{1,4} = 0.21$, $P = 0.7$, Fig. 4).

Size and sex combs

Males from the low and high lineages did not differ in either size (mean ± SE: 1.73 ± 0.006 vs. 1.74 ± 0.005 mm for the low and high lineages, respectively; $F_{1,4} = 0.13$, $P = 0.7$) or number of sex comb bristles (11.16 ± 0.12 vs. 11.44 ± 0.14 for the low and high lineages, respectively; $F_{1,4} = 0.5$, $P = 0.5$).

### Mating success with mature females

Under no choice (1 male + 1 female), males from the high lineages had a non-significant, higher mating success than males from the low lineages (Wald $\chi^2_1 = 3$, $P = 0.08$; Fig. 5A). Under mate choice (1 low male + 1 high male + 1 female) however, males from the high lineages had a strong mating advantage over males from the low lineages (Wald $\chi^2_1 = 60.4$, $P < 0.001$; Fig. 5B). Male color had no effect on mating success (Wald $\chi^2_1 = 0.37$, $P = 0.5$).

Longevity

Males from the low and high lineages had similar lifespans ($F_{1,4} = 0.07$, $P = 0.8$) but males housed with females had shorter lifespan ($F_{1,229} = 33.9$, $P < 0.001$; Fig. 6). The interaction between the selection (low and high) and female treatment was not significant ($F_{1,229} = 0.17$, $P = 0.7$).

Effects of relaxed selection

Relaxed selection for four generations had a small, non-significant effect on forced copulation rates (selection type by treatment interaction: Wald $\chi^2_1 = 0.5$, $P = 0.4$), which were about twice as high in the high than low forced copulation lineages (Wald $\chi^2_1 = 10.4$, $P < 0.005$; Fig. 7).
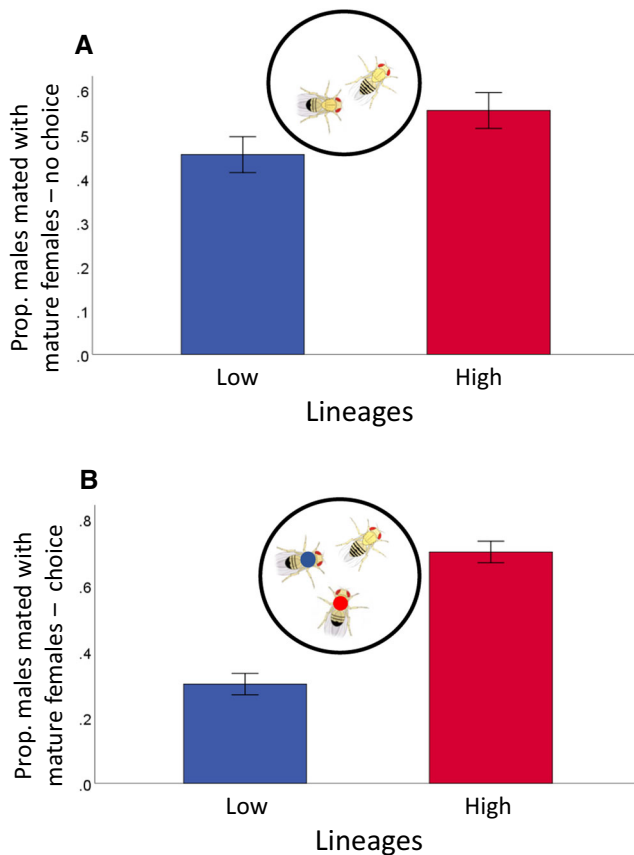
Figure 5. Mating success (±1 SE) of males from the low (blue) and high (red) lineages (A) when each male was paired with a female (no choice; *N* = 300 trials), and (B) when one male from a low and one male from a high lineage competed for mating (choice; *N* = 600 trials). We calculated means and SEs from the raw data.

Discussion

Our major findings were that we could rapidly select for forced copulation success (Fig. 1C), and that males from the high forced copulation lineages were indeed more sexually aggressive toward teneral females as indicated by the longer durations they spent pursuing and mounting these females (Fig. 2). Males from the high forced copulations lineages, however, were not more sexually aggressive toward either non-teneral, conspecific females or mature, heterospecific females (Fig. 3), and did not show higher rates of male-male aggression (Fig. 4). Finally, the low and high lineages did not differ in either body size or number of bristles in their sex-comb. While we expected to find trade-offs associated with sexual aggression, males from the high forced copulation lineages actually had a higher mating success with mature females, especially under male-male competition (Fig. 5), and showed no elevated mortality rates when housed with females (Fig. 6). Furthermore, relaxed selection for four generations did not significantly reduce the difference in sexual

aggression between the low and high lineages (Fig. 7). We discuss these results below.

The divergence of the low and high forced copulation lineages (Fig. 1C) is consistent with our recent quantification of heritable variation in forced copulation success (Baxter et al. 2019). Our choice of experimental design for the artificial selection experiment, however, has clear implications for what we can infer from the experiments on the evolved lineages. Each generation, after selecting males based on their forced copulation success, we had to pair them with unselected, mature females from their own lineage in order to generate the next generation. Hence, selection could occur owing to differential paternity success at this reproductive stage. We had carefully considered our options for this stage. At the two extremes, we
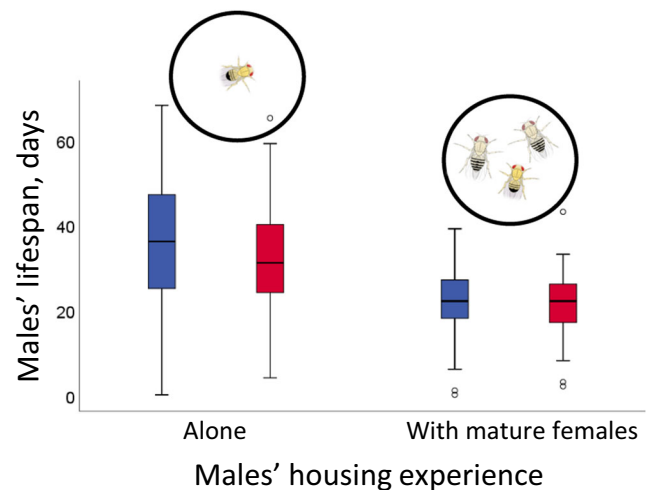
Figure 6. The lifespan of males from the low (blue) and high (red) lineages when each male was housed either alone or with two females. *N* = 237 trials.
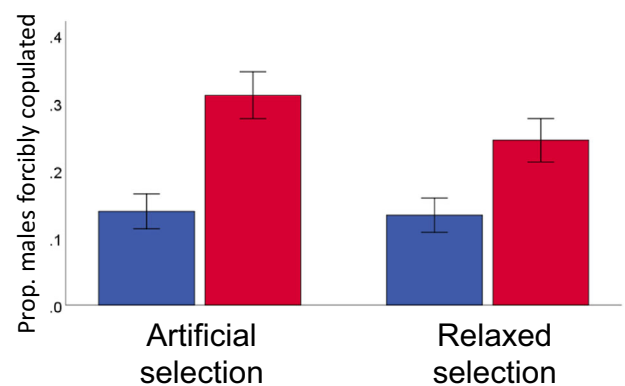
Figure 7. The proportion (±1 SE) of males from the low (blue) and high (red) lineages that forcibly copulated with teneral females. Lineages were subjected to either 19 generations of artificial selection, or 15 generations of artificial selection followed by four generations of relaxed selection. *N* = 720 trials. We calculated means and SEs from the raw data.

could either place the 48 selected males from each lineage with 48 unselected, mature females from their lineage, or place each selected male with a single unselected, mature female from their lineage. The former could generate strong sexual selection owing to differential paternity success while the latter would have fully eliminated sexual selection. We rejected both of these options. We had no reason to generate strong sexual selection but also had a strong argument against eliminating it altogether. The problem with eliminating sexual selection is that it could have selected, in the low lineages, for males with a low propensity to pursue or mate with any female. That is, instead of selecting specifically for forced copulation success, we might have selected for low quality males with poor mating success. As a compromise, we placed each group of four selected males with four unselected mature females from their lineage. Apparently there is no strong genetic correlation between forced copulation success and paternity success with mature females because we could select for low forced copulation success in males that subsequently were under pre- and post-copulatory competition for paternity.

While we documented rapid evolution of forced copulation success in our lineages, there was relatively little change in the latter half of the experiment (Fig. 1C). The lack of a downward trend in the low lineages in the latter generations could indicate that some teneral females are susceptible to forced copulation even after relatively short and moderate male pursuit. The apparent ceiling in the high lineages is more difficult to explain. We hypothesize that a fair proportion of teneral females can thwart males' forced copulation attempts. That is, while our current work focuses on the males' component of sexual aggression toward females, future work should investigate mechanistic and functional aspects of heritable variation in teneral females' ability to resist forced copulation. Finally, we should note that, while we did not subject teneral females to artificial selection, selection in the baseline laboratory population may have increased teneral females' resistance to forced copulation over time. In general, it is well established that a variety of traits evolve rapidly in animals brought from the field into the laboratory (Sgro and Partridge 2000; Simões et al. 2007; Hoffmann and Ross 2018).

Our behavioral observations indicated that the difference in sexual aggression between males from the low and high lineages was confined to their behavior toward teneral females (Fig. 2). Both the pursuit and mountings of non-teneral, conspecific females and mature, heterospecific females were similar between the low and high lineages (Figs. 3 and S2). That is, we did not select for general sexually aggressive traits expressed indiscriminately toward all females. Previous results from our laboratory agree with this conclusion. Wild type Canton S males performed many more mounting attempt towards teneral than non-teneral females (Fig. 6 in Seeley and Dukas 2011). In general, different female categories (e.g., teneral, immature non-teneral, mature

virgin, or mature recently mated) provide distinct feedback to pursuing males, meaning that males can possess abundant information for establishing their distinct pursuit strategies (Dukas and Scott 2015).

In the experiments with non-teneral and heterospecific females, we also critically tested for possible differences in persistence between the males from the low and high lineages by comparing their pursuit of females during the first and last 15 min of the 1 h trials. Males from the high lineages, however, showed no higher persistence in either experiment (left vs. right bars in Fig. 3). Hence, our results indicate that there is no strong genetic correlation between males' coercive behavior and non-aggressive pursuit of females. In other words, males' coercive behavior is not an unavoidable byproduct of their naturally high motivation to mate.

Similar to the distinction between the pursuit of teneral and non-teneral females, our results showing no difference in male-male aggression between the low and high forced copulation lineages (Fig. 4) indicate that aggression in one context is not necessarily genetically linked with aggression in another context. While the function of both types of aggression is mating, selection on males' eagerness to coerce teneral females did not select for eagerness in defending a resource that would be attractive to prospective mates. In a well cited paper, Moyer (1968) suggested that the classical treatment of aggression as a unitary trait is inappropriate and that researchers should focus instead on distinguishing among mechanistically independent types of aggression. Although Moyer's argument has been widely accepted (Archer 1988; Huber and Kravitz 2010; Asahina 2017), there are still limited data and no consensus about the genetic correlation among types of aggression. Given fruit flies' prominence as a model system in both neurogenetics and evolutionary biology, further research such as ours can shed light on this important issue, which is relevant to all species (Veroude et al. 2016; Wrangham 2018; Wrangham 2019).

While our artificial selection protocol could lead to changes in any type of trait, our data indicate a specific change in behavior related to pursuing teneral females. Neither body size nor sex comb structure changed in the evolved lineages. There is ample genetic variation in both body and sex comb size (Partridge et al. 1994; Ahuja and Singh 2008; Turner et al. 2011), which could lead to rapid evolutionary changes. There are two possible explanations for this lack of change. First, variation in body and sex comb size may not be associated with forced copulations success. We have no data pertinent to this possibility. Second, sexual selection during the mating phase of our experimental protocol could have been the dominant factor maintaining similar body and sex comb sizes in the low and high lineages. It is indeed known that both body and sex comb size play a role in males' mating success with mature females (Cook 1977; Partridge and

Farquhar 1983; Dukas 2005; Hurtado-Gonzales et al. 2015). Nevertheless, while we measured two pertinent morphological traits, there may be other, more important characteristics that determine forced copulation success, for example, strength or genital structures.

Compared to males from the low lineages, males from the high lineages had non-significant, 22% higher mating rates under no choice, and significant, 133% higher mating rates under mate choice (Fig. 5). We do not know what features determined the higher mating success of males from the high lineages. Our video scorings of males from the low and high lineages indicated no difference in pursuit and mountings (Figs. 3A and S2A). However, there may be other subtle behavioral differences between the low and high lineages, or the differences may be in non-behavioral traits such as cuticular hydrocarbons, which we have not measured. It is possible that the large differences in mating success between the low and high lineages under mate choice resulted from agonistic male-male interactions such as courtship interference (Baxter et al. 2018). Overall, the higher mating success of males from the high than low lineages indicates a positive genetic correlation between forced copulation success and mating success with mature females. This appears to contradict our earlier conclusion that there is no strong genetic correlation between forced copulation success and mating success with mature females, which was based on both our success at artificial selection and the distinct male behavior toward teneral and non-teneral females (Figs. 1–3). While we have no resolution at this point, we hope that our ongoing genomic work and further behavioral experiments will clarify this issue.

The natural levels of forced copulation success in our initial population were about 20% and we had previously recorded similar levels in another wild, outbred population (Seeley and Dukas 2011). While we could decrease and increase the levels of forced copulation via artificial selection (Fig. 1C), it was sensible to assume that the observed natural levels represented a trade-off between fitness costs and benefits. Our data indicated low mating success with mature females in the lineages selected for low forced copulation success (Fig. 5). Hence, this represents a fitness cost linked to low forced copulation success. Given the link between low forced copulation success and low mating success with mature females, one would predict that relaxed selection would result in rapid increase in forced copulation success in the low lineages. Surprisingly though, this did not happen after four generations of relaxed selection (Fig. 7). It is possible that four generations were insufficient especially if there is only moderate genetic correlation between forced copulation success and mating success with mature females.

As for the cost associated with the evolution of high forced copulation success, we hypothesized that males in the high lineages might incur higher mortality rates associated with the greater cost of pursuing low-receptivity females such as teneral and recently mated females. Male pursuit of females is known to reduce their longevity (Cordts and Partridge 1996). Furthermore, to succeed in both forced copulation with teneral females and mating with recently mated females, males have to spend much longer times in pursuit than when paired with mature, virgin females (Seeley and Dukas 2011; Baxter and Dukas 2017). Because females in our longevity experiment were previously mated for much of their 2-week period of housing with each focal male, we predicted that longevity would be lower in males of the high than low lineages. We found, however, no such pattern (Fig. 6). Similarly, we documented no convergence in forced mating success in the lineages subjected to four generations of relaxed selection (Fig. 7). While we documented no costs associated with high forced copulation success, it is possible that our experiments did not capture some realistic settings where males from the high lineages would incur fitness costs. For example, it is possible that males that spend more time pursuing teneral females lose opportunities to pursue and mate with receptive, mature females. Similarly, owing to sperm and seminal fluid limitations, forced matings with teneral females might decrease males' paternity success with mature females.

In conclusion, our artificial selection experiment generated low and high forced copulation lineages that showed distinct sexually aggressive behaviors toward teneral but not mature females. While this indicates no strong association between behaviors directed toward teneral and mature females, our data also indicated a positive genetic correlation between forced copulation success with teneral females and mating rates with mature females under competition. We hope that our current genomic work on the evolved lineages will help clarify the complex association between forced and consensual mating success.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Statistical table for the GLMM of the 20 generations of artificial selection (Figs 1 and S1).
Fig. S1. The percentage of males from each of the 3 low (blue hues) and 3 high (red hues) lineages that forcibly copulated with teneral females over 20 generations of selection.
Fig. S2. The proportion of time males from the low (blue) and high (red) lineages spent mounting (A) non-teneral conspecific females (N=85 trials), and (B) heterospecific, *D. simulans* females (N=82 trials)