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# Female-limited X-chromosome evolution effects on male pre- and post-copulatory success

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Intralocus sexual conflict arises when the expression of shared alleles at a single locus generates opposite fitness effects in each sex (i.e. sexually antagonistic alleles), preventing each sex from reaching its sex-specific optimum. Despite its importance to reproductive success, the relative contribution of intralocus sexual conflict to male pre- and post-copulatory success is not well-understood. Here, we used a female-limited X-chromosome (FLX) evolution experiment in *Drosophila melanogaster* to limit the inheritance of the X-chromosome to the matriline, eliminating possible counter-selection in males and allowing the X-chromosome to accumulate female-benefit alleles. After more than 100 generations of FLX evolution, we studied the effect of the evolved X-chromosome on male attractiveness and sperm competitiveness. We found a non-significant increase in attractiveness and decrease in sperm offence ability in males expressing the evolved X-chromosomes, but a significant increase in their ability to avoid displacement by other males' sperm. This is consistent with a trade-off between these traits, perhaps mediated by differences in body size, causing a small net reduction in overall male fitness in the FLX lines. These results indicate that the X-chromosome in *D. melanogaster* is subject to selection via intralocus sexual conflict in males.

## 1. Introduction

Sperm competition theory [1] predicts that males partition their energy investment between pre- and post-copulatory success when resources are limited, leading to a trade-off between sexual attractiveness and sperm competitiveness [2,3]. Selection on pre- and post-copulatory success in males can also lead to sexual conflict, because traits that improve male paternity success can reduce the fitness of their mates [4,5]. *Interlocus* sexual conflict (IRSC) arises when these sex-specific strategies are mutually incompatible [6]. Reproductive proteins that prevent selective sperm use by females and increase male fertilization success relative to rivals at a cost to female fecundity are a classic example of interlocus sexual conflict in *Drosophila* [7,8].

By contrast, *intralocus* sexual conflict (IASC) can arise when the expression of shared alleles at a single locus generates opposite fitness effects in each sex (i.e. sexually antagonistic alleles) [9], preventing them from reaching their respective optima [10]. The unequal inheritance pattern of the X-chromosome between the two sexes has led theoretical models to predict an accumulation of sexually antagonistic alleles on the X-chromosome [11]. Some empirical data support this model in *Drosophila melanogaster* [11–13], but not all [14,15].

IASC and IRSC are two distinct forms of sexual conflict that can interact over a given trait (e.g. sperm competitiveness), since IASC may constrain
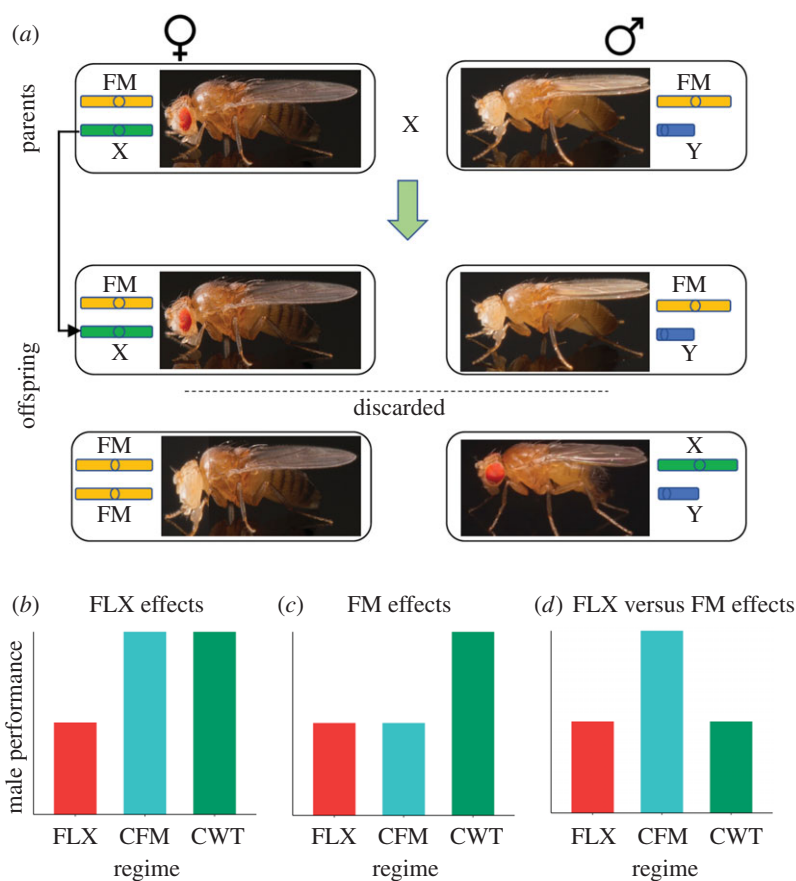
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**Figure 1.** Protocol for the female-limited X-chromosome (FLX) evolution experiment and graphical interpretation of the selection regimes' effects on male performance. (*a*) The FLX evolution protocol. The evolving X-chromosome (green bar) is passed from mother to daughter with the help of an FM balancer chromosome (yellow bar). The parental cross produces four genotypes, of which the offspring above the dashed line are crossed to produce the next generation, and the offspring under the dashed line are discarded. At generations 107, 143 and 160, males expressing the evolved X-chromosome (male offspring under the dashed line) were used to study male pre- and post-copulatory success. The FM balancer carries several phenotypic markers, which can be used to phenotype offspring, as illustrated by the pictures next to the genotypes (fly pictures by Qinyang Li). (*b,c,d*) Potential outcomes of the experiments. (*b*) FLX effects. If FLX males perform worse than CFM and Cwt males, this should be a result of the FLX selection. (*c*) FM effects. If FLX and CFM males both perform worse than Cwt males, this suggests an effect of the FM balancer. (*d*) FLX versus FM effects. If males from the FLX regime perform worse than CFM males but similarly to Cwt males, this suggests that the deleterious effects of a feminized X-chromosome seem to outweigh the FM effect.

the response to IRSC [16,17]. How IASC contributes to IRSC is still not well-understood [16,18], although there is evidence that sexually antagonistic loci may often be sex-biased to some extent [19,20]. In this study, we attempted to investigate how IASC affects male reproductive success using a female-limited X-chromosome (FLX) evolution experiment, which has resulted in feminization of body size and development time in males [15]. After more than 100 generations of FLX evolution, we measured three male fitness components and analysed how male pre- and post-copulatory success responded to the FLX evolution experiment. We expected to see a reduction in both pre- and post-copulatory fitness components in males expressing a feminized FLX X-chromosome, assuming that the feminization we observed in other traits is detrimental to male-specific fitness (e.g. via pleiotropic effects, or if body size trades-off with male-limited reproductive traits).

## 2. Material and methods

### (a) Fly stocks

All experimental populations were established from a large outbred LH$_M$ stock population and kept under standard conditions (25°C; 12 h L : 12 h D light–dark cycle; 60% humidity; cornmeal–molasses–yeast food [21]). The experimental protocol for the evolution experiment is described in full detail in Lund-Hansen *et al.* [15]. Briefly, an FM balancer (FM7a) was used to control and limit the inheritance of the selected X-chromosomes to the matriline in the female-limited X-chromosome (FLX) selection regime (figure 1*a*). We also included two control regimes: (i) Control FM (CFM), which was added to control for any unexpected effects of the FM balancer, and (ii) Control wild type (Cwt), which controls for other aspects of the experimental protocol. The three regimes were kept in four replicate populations each (see electronic supplementary material).

All target X/Y males carried the evolved genome (X, Y and autosomal chromosomes) and were collected directly from the selection regimes (figure 1*a*, male offspring under the dashed line) using light $CO_2$ anaesthesia (see electronic supplementary material).

### (b) Male attractiveness

We measured male attractiveness as copulation latency (the time for a female to accept a mating) and mating frequency (the frequency with which the target male is accepted). One 5-day-old virgin LH$_M$ female was paired with a 5-day-old target virgin male. The female mating responses were observed over a period of 30 min and the time until copulation (copulation latency), and whether it occurred at all (mating frequency)

were noted. This experiment was carried out at generation 107, in 12 blocks within a day, with 20 flies per selection regime per replicate population in each block, estimating the attractiveness of a total of 80 males per regime (see electronic supplementary material).

## (c) Sperm competition

Sperm competitiveness of *D. melanogaster* males is defined by both the ability to displace sperm that is resident in the female's reproductive tract (sperm offence) and the ability to resist displacement by sperm from subsequent males (sperm defence) [1,22]. Details of the protocol can be found in the electronic supplementary material. Briefly, virgin females are provided with the opportunity to mate with a target or competitor male on consecutive days. For sperm defence, the target male is the first to mate, and for offence, the target male is the second to mate. Competitor males carry a brown eye mutation ($bw^-$) while target males are wild-type. Because the wild-type red eye colour is dominant over the brown, the number of target and non-target offspring produced by the female is used as a measure of paternity share.

Both the sperm defence and offence experiments were carried out at generation 143, in 15 experimental replicates per regime per replicate population. Females that produced no offspring after the first mating were removed from the experiments. To ensure sufficient sample sizes, we performed the offence experiment again at generation 160, with an average of 26 experimental replicates per regime per replicate population.

## (d) Statistical analysis

All statistical analyses were conducted in RStudio v. 1.1.463 (http://www.rstudio.com/). The mating frequency was analysed by fitting a generalized linear mixed-effects model (GLMM) from the R package *lme4* [23], with the family set as binomial (mated/not mated), with regime and block as fixed factors, and with replicate population nested within the regime as a random effect. Copulation latency was analysed using the same model (GLMM), but the family set as gamma. These two models were tested for overdispersion using the *dispersion_glmer* command in the *blmeco* package [24].

For both the sperm defence and offence analyses, we first excluded all vials that had fewer than 10 offspring, then we used *cbind()* to create a response variable matrix of successes (number of first male's offspring) and failures (second male's offspring) as the dependent variables in a binomial zero-inflated GLMM (*glmmTMB* package in R) [25]. Regime and block (for offence) were fixed factors, and replicate population was nested within regime as a random effect. We checked for overdispersion by visual inspection of the plot of scaled residuals using the *simulateResiduals()* function in the *DHARMa* package [25]. The *Anova* command from the *car* package [26] was used for type 3 sums of squares significance testing. *Post hoc* analysis of effects was performed using the package *emmeans* [27].

Finally, to see if the differences we observed could be related to changes in body size, we used Pearson correlations to analyse the relationship between male body size (thorax size from [15]) and sperm competitive ability (both defence and offence), with population means as the unit of analysis [28].

## 3. Results and discussion

Limiting the inheritance of the X-chromosome to females for over 100 generations should allow X-linked female-beneficial alleles to increase in frequency, and we have previously found evidence of feminization of body size and development time

in the FLX selection regime [15]. We therefore expected that expressing the experimentally evolved X-chromosomes would result in a reduction in pre- and/or post-copulatory fitness components in males, assuming phenotypic feminization is detrimental to males. Our results were consistent with this expectation for sperm offence, although the effect did not quite reach significance, but not for an attractiveness or sperm defence. We did not find any significant correlations between body size and sperm offence or defence on the replicate population level (electronic supplementary material, figures S1 and S2), but this may not reflect patterns on the individual level.

We found no significant difference between regimes in either mating frequency ($p = 0.24$, electronic supplementary material, table S1) or copulation latency ($p = 0.48$, electronic supplementary material, table S1) in the no-choice attractiveness experiment. This indicates that the FLX males were not refused more or less often than the control males (figure 2*a*) and were not more or less attractive than the control males (figure 2*b*), despite their expression of the evolved X-chromosome. If anything, FLX males were actually slightly more attractive than the other males (figure 2*a,b*), which is counter to our expectations, even if the difference is not significant. However, we have previously found that FLX males are larger than the other males [15], and being large increases male attractiveness and success in intrasexual competition for access to females [29,30]. This may have compensated for possible decreases in attractiveness in other traits (e.g. courtship behaviour), if any exist.

For post-copulatory success, we found a significant difference between selection regimes in sperm defence ($p = 0.009$), where FLX males sired a larger proportion of the offspring than both CFM and Cwt males (figure 2*c*, table 1). This increase is attributable to the selection regime and not the presence of the FM balancer (figure 1*b*) and is consistent with the previously observed increase in body size, if larger males transfer more sperm or are preferred under cryptic female choice [6,31]. By contrast, there was a trend towards a significant difference between selection regimes in sperm offence ($p = 0.08$, table 1), where CFM males had higher performance in sperm offence than FLX males, which sired a similar proportion of offspring to that of Cwt males (figure 2*d*). The (non-significant) increase in sperm offence seen in CFM males is likely to be a result of autosomal and/or Y-linked adaptation to regain fitness in the presence of the balancer (figure 1*d*). However, the decreased offence ability in FLX males compared with CFM males suggests that any adaptation to regain fitness in the presence of the balancer seems to have been outweighed by the deleterious effects of a feminized X-chromosome in FLX males (figure 1*c*). This interpretation is supported by our previous finding that males carrying an FM balancer have substantially lower fitness than wild-type males [15]. We are currently carrying out follow-up experiments to disentangle these effects.

From previous work, we know that CFM males have a marginally higher total reproductive fitness than FLX and Cwt males [15]. Although this difference was not significant, the overall pattern was very similar to the pattern seen here in sperm offence. Collectively, these results suggest that under normal conditions, any potential increase in attractiveness or sperm defence in FLX males is outweighed by other negative effects, including in sperm offence. In addition, other research has shown that large males are
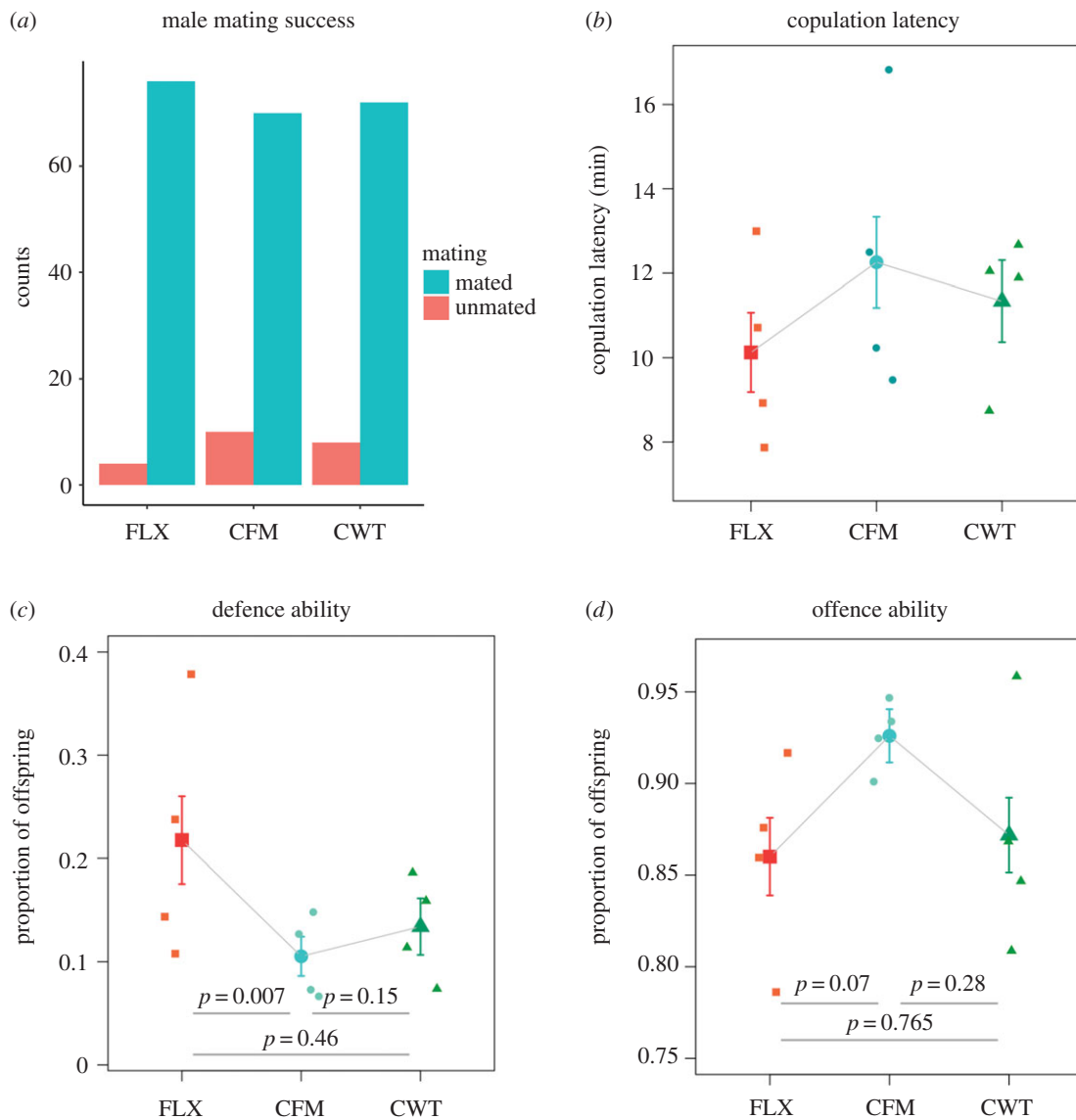
**Figure 2.** Male mating attractiveness and sperm competition. (*a*) Counts of mated (blue) and unmated (red) males in the mating experiment for the three regimes. (*b*) Comparison of copulation latency (mating speed) in minutes between regimes. (*c*) Sperm defence ability measured as proportion of offspring sired by the target male. (*d*) Sperm offence ability measured as proportion of offspring sired by the target male. Points with error bars represent overall means and standard errors, and individual points are the means for the four replicate populations. FLX: red, CFM: blue, CWT: green. *p*-values are from Tukey's HSD test.

**Table 1.** Summary of the results from ANOVA analysis of GLMM models.

| fixed effects | | $\chi^2$ | d.f. | *p*-value |
|---|---|---|---|---|
| defence | intercept | 53.36 | 1 | <0.001 |
| | regime | 9.52 | 2 | 0.009 |
| offence | intercept | 41.6 | 1 | <0.001 |
| | regime | 5.05 | 2 | 0.08 |
| | block | 198.77 | 1 | <0.001 |

more harmful to females [32,33]. This may also serve to decrease the post-copulatory success of the larger FLX males.

Several general conclusions can be drawn from our results. First, that IASC may affect sex-specific fitness in complex ways. We expected that release from intralocus sexual conflict by a feminization of the X-chromosome would result in a decrease in some or all male fitness components, under the assumption that feminization is detrimental to

male fitness. Instead, we found evidence of an increase in sperm defence ability in FLX males. This illustrates how difficult it can be to predict the outcome of releasing one sex from IASC, which has also been shown in *Timema* stick insects, where a transition to asexuality actually resulted in masculinization of the female expression profile [34]. Second, the fact that sperm offence changed both in CFM males relative to Cwt males, and in the FLX males relative to the CFM males, suggests that this trait has been subject to conflicting selection pressures under our selection regimes. Specifically, the presence of the balancer seems to have resulted in compensatory evolution on the Y-chromosome and autosomes. Finally, although we cannot be sure of the exact genetic mechanism, our results are also suggestive of a trade-off between pre- and post-copulatory traits as predicted from sperm competition theory, specifically that an investment in increased body size may come at a cost to sperm offence ability (electronic supplementary material, figure S2). This type of effect (but in the opposite direction) was seen in Prasad *et al.* [28] and suggests that the large but fast-developing FLX males [15] may trade-off body size

against gonad development [15,35,36], although further investigation is needed.

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