

Rapid male benefit adaptation in response to sex-limitation, mates, and genetic context in *Drosophila melanogaster*

Harshavardhan Thyagarajan^{1,2}, Mindy G. Baroody^{1,3}, Imran Sayyed¹, Joshua A. Kowal¹, Troy Day¹, and Adam K. Chippindale¹

Author institutional affiliations

1 – Department of Biology, Queen's University, Kingston, Ontario, Canada

2 – Department of Biology, University of Fribourg, Fribourg, Switzerland

3 – Department of Medicine, McMaster University, Niagara, Ontario, Canada

Corresponding author: Harshavardhan Thyagarajan <harshavardhanthyagarajan@gmail.com>

Supplementary material

Supplementary material is available online at *Evolution*.

Data availability

Data has been archived on Dryad at DOI: [10.5061/dryad.r2280gbv8](https://doi.org/10.5061/dryad.r2280gbv8)

Author contributions

HT, AKC and TD developed the overall theme of the study; HT and AKC designed the experiments; HT, AKC, IS, MGB, & JAK carried out the experiments; HT analysed the data; HT wrote the first draft of the paper; HT and AKC contributed to the final draft and revisions.

Funding

Funding for the work was from a NSERC Discovery grant (RPGIN-2018-06462) to AKC & TD.

Conflict of interest

The authors declare no competing interests.

Acknowledgments

Several undergraduate students were involved in lab maintenance and data collection during the period of this study. We thank Avery Want, Halle McNamara, Ronni Prince, Katelyn Viau, Alex Montenegro-Monreal, and Simmal Grewal for their efforts. **AI Usage:** LLM assistance was used to improve clarity, grammar, and readability of manuscript text in response to review comments, as well as edit and troubleshoot analysis code. The model used was GPT-5.5 (OpenAI, San Francisco, CA, USA), accessed via ChatGPT web interface. Applications included natural-language prompts for editing, rephrasing, and shortening author-generated text; and for code debugging and modification of existing analyses. The authors reviewed and verified all outputs and take full responsibility for the final manuscript, analyses, and conclusions.

ABSTRACT

Intralocus sexual conflict (IaSC) arises when selection favours different trait values in females and males despite their shared genetic architecture. In the presence of IaSC, sex-limited selection is expected to resolve this conflict by allowing one sex to adapt without constraint from selection acting on the other sex, with intersexual genetic correlations predicted to drive a decline in the fitness of the opposite sex. In a recent study, we found that male-limited (ML) selection produced the predicted decline in female fitness, but male fitness improvement was sensitive to genetic background, suggesting unintended sources of adaptation. Here we dissect these evolutionary responses using a fully factorial assay spanning control-like and selection-like conditions, explicitly accounting for the unusual genetic features of the clone-generator (CG) breeding system and the specialized (CG) females used to enforce male-limited inheritance of nuclear genetic variation. Evolved male fitness increased by more than 100% (relative to controls) when tested with all the features of the CG breeding system. Retaining the CG genetic background while replacing CG females with control females yielded an approximately 66% fitness increase. In a control-like genetic background, male fitness increased by 26% when males competed for the CG females they had potentially adapted to, but only by a marginal 6% otherwise. Despite this strong context dependence, the underlying phenotypic responses were remarkably consistent: selected male improvements were driven by enhanced precopulatory traits; greater attractiveness, reflected in higher mating success and shorter mating latency; while postcopulatory traits, including sperm offense (P2) and fecundity induced in mates, remained unchanged. Intriguingly, evolved males also inflicted substantially less mate harm on CG females, contrary to findings from an earlier ML selection experiment. Together, these findings provide clearer evidence of intralocus sexual conflict, expressed as male-benefit adaptations alongside previously established costs to females, but demonstrate that these adaptations were also shaped by the genetic manipulations and mating context of the experimental design.

Keywords

Intralocus sexual conflict, experimental evolution, *Drosophila melanogaster*

INTRODUCTION

Gonochoric organisms experience fitness costs when sex-specific selection acts on shared traits, a phenomenon termed intralocus sexual conflict (IaSC). As sex-specific selection is common and traits are often genetically correlated between sexes, IaSC is expected to be widespread (Lande 1980; Poissant *et al.* 2010). It is predicted to influence: (1) maintenance of genetic variance for fitness through balancing or disruptive selection (Connallon & Clark 2014, but see Flinham *et al.* 2025), (2) evolution of sexual dimorphism (Lande 1980), and (3) patterns of selection upon sex-biased gene expression (Connallon & Knowles 2005, Wright *et al.* 2018).

IaSC has been studied using a variety of approaches including comparative biology, quantitative genetic analyses, genome-wide association, and experimental evolution with model organisms (Bonduriansky & Chenoweth 2009). *Drosophila melanogaster* features prominently, particularly with the analysis of intersexual genetic correlations (e.g., Chippindale *et al.* 2001), and “sex-limited” evolution (e.g., Rice 1998, Prasad *et al.* 2007) revealing evidence of a negative association between genetic variation favoured in one sex and its effects upon the other. Sex-limited experimental evolution has served as a key experimental tactic within this body of work. These studies use a variety of genetic markers and aberrations to restrict chromosome transmission to a single sex (typically males), allowing variation to respond exclusively to selection in that sex. Such responses can be expressed through different components of sexual selection: pre-copulatory selection, which acts through traits affecting mating success, and post-copulatory selection, which acts after mating through processes such as sperm competition and ejaculate-female interactions. If IaSC is present, fitness gains in the selected sex should come at the expense of performance of the opposite sex, through shifts in allele frequencies at sexually antagonistic loci.

A classic design for sex- (male-) limited selection in *D. melanogaster* is the “clone-generator” (CG) system developed by Rice (1996). In this breeding scheme, a combination of visible markers and genetic aberrations (a compound-X chromosome and translocated major autosomes) allow patrilineal transmission of the X-chromosome, matrilineal transmission of a Y chromosome, and easy sorting of paternal autosomal haplotypes that are transmitted from father to son (fig. S1a) whilst the maternally-derived vehicles of their transmission are discarded each generation. In other words, whole genomic haplotypes - “hemiclones” - can be bred into many copies as sperm and fertilize eggs that develop into males. With large populations of genetically diverse hemiclones, selection can occur exclusively on male function, with the clone-generator females used only for propagation.

Rice (1996) showed that males could rapidly adapt to their evolutionarily “arrested” mates, enhancing reproductive success and competitive ability while also increasing harm to these females, as reflected in higher mortality - likely a collateral effect of post-copulatory sexual selection. Rice (1998) later demonstrated that these fitness gains were generalizable and not entirely specific to CG females. Further, females expressing the male-limited evolved haplotypes

exhibited slower development, which was interpreted as an indication of reduced fitness associated with male fitness gains (i.e., intralocus sexual conflict – IaSC). In a subsequent male-limited evolution (ML) experiment, Prasad *et al.* (2007) expanded the analysis of IaSC and confirmed the slower development of ML-expressing females. Here, they attributed the evolutionary response more explicitly to the optimization of male development, as males too slowed down under ML-selection. Sexual dimorphism in development time and body size is highly conserved in this species and remained unchanged under ML-selection. Prasad *et al.* (2007) explained these changes as a shift in the direction of extant dimorphism, with both sexes displaying reduced growth rate (later adult eclosion and smaller body size), or “masculinization” of development. Later work (Bedhomme *et al.* 2008; Abbott *et al.* 2010) showed that these smaller, slower-developing males were more symmetrical, more male-like in phenotype, and more attractive to mates.

Important differences between these two experiments’ outcomes emerged upon comparison, leaving key aspects of ML-selection responses unresolved. While Rice (1996, 1998) emphasized responses in post-copulatory traits such as sperm competition and mate harm, Prasad *et al.* (2007) and Jiang *et al.* (2011) did not corroborate these findings despite using the same LH_M base population and a larger experimental scale. These contrasting readouts and the predominant use of LH_M in the *Drosophila* sexual conflict literature suggested to us the need for investigation of additional fruit fly populations.

We recently reported on such an experiment using the male-limited (ML) selection approach with different populations of *D. melanogaster* (Thyagarajan *et al.* 2025). Our approach to assaying the response was to remove the genetic and mating context of the ML selection protocol, in an attempt isolate responses to masculinizing selection from the selection context. Consistent with IaSC predictions, females expressing ML-evolved chromosomes displayed a substantial reduction in fitness. Female performance could also decline from the atrophy of sex-limited genes when selection is only operating on males, but fitness decline was rapid and consistent across replicate populations; this pattern, also observed by Prasad *et al.* (2007), would not be expected from stochastic process like mutation accumulation. In contrast with previous studies, we detected only a marginal increase in male fitness in selected lines relative to controls. However, an assay of heritable variation in male reproductive fitness revealed a pronounced reduction in variance accompanied by a marked increase in selected male fitness. Importantly, this finding emerged from a quantitative genetic assay with test animals that deviated solely in the incorporation of a CG-derived Y chromosome, suggesting that male fitness had indeed improved, albeit in a manner contingent on assay conditions. These mixed results motivated a factorial examination across test conditions to characterise the context-dependent expression of male fitness improvement.

One explanation for the context-dependent responses observed under ML selection is that features of the experimental system shape the ML selection environment. Here, we explicitly

take into consideration the fact that the CG females used to passage selected haplotypes are not passive components of the breeding design, but can themselves influence evolutionary outcomes.

First, the repeated introduction of CG females as mates from a genetically distinct population disrupts the coevolutionary arms race between the sexes, creating an evolutionarily arrested sexual-selection and conflict environment (*sensu* Rice 1996). Specifically, through the many genetic aberrations the CG population carries, this is an environment characterized by females that are particularly susceptible to persistent male courtship and mating (personal observation), exerting little control over mating-rate conflict.

Second, the very same genetic aberrations that enable ML selection also constitute a distinctive genomic and cytoplasmic background that could potentially shape selection outcomes. For example, the translocation of the two major autosomes (t2;3), used to facilitate handling by forcing co-segregation of selected wild-type chromosomes, could be a target of adaptation. While all the visible markers are recessive, this does not assure that the aberration has no fitness impact, as has been assumed in some earlier studies (Rice 1996; Prasad *et al.* 2007). Next, clone-generator (CG) females possess an unusual X-XY karyotype, carrying a compound X chromosome alongside a Y chromosome. This arrangement reverses sex chromosome parent of origin: sons are produced when X-carrying sperm fertilize Y-carrying eggs. If X-chromosomes are normally modified epigenetically for daughters, sons inheriting an X from their father could experience reduced fitness. And despite limited coding content, the Y chromosome can exert broad regulatory effects (Chippindale & Rice 2001; Lemos *et al.* 2008; Jiang *et al.* 2010) and is known to have such influences even when expressed in X-XY females (Branco *et al.* 2017). Finally, CG females supply a foreign cytotype: a constraint that cannot be removed through backcrosses because the compound-X is inseparable from its cytoplasmic background. Strict maternal mitochondrial inheritance favours female-benefit variants, even when harmful towards male function (Cosmides & Tooby 1980, Frank & Hurst 1996, Gemmell *et al.* 2004, cf. Keaney *et al.* 2019). This "mother's curse" may result in compensatory adaptation by nuclear loci to restore male fitness (reviewed in Dapper *et al.* 2023).

Here we follow up on Thyagarajan *et al.* (2025) to fully characterize the extent of adaptation in our male-limited evolution experiment. We assayed selected and control males for mating success, fecundity induction (ability to upregulate female fertility), offspring sex-ratio, and sperm offense (P2). These traits were assayed in a fully factorial design across two genetic backgrounds: a complete clone-generator (CG) background retaining all selection features, and a control-like (C) background lacking them; and against both CG and recessive marked control (C) females, with males in competition with marked control males. We additionally quantified mate harm imposed by selected and control males (in both genetic backgrounds) on CG females, given the potential for this sexual selection environment to influence male fitness through reduced female resilience to mating stress. Finally, we evaluated the individual fitness effects of three components of the selection system: foreign cytotype, the female-derived Y chromosome, and the translocated autosomes.

METHODS

Source populations, Selection treatment

The source populations, selection protocols and controls are described in detail in Thyagarajan *et al.* (2025). Briefly, replicate pairs of populations – a male-limited (ML) and a matched control (MC) population – were each derived from three independent lab-adapted populations that had been maintained separately since their derivation from the IV base population in 1980 (Rose 1984). To facilitate comparison with previous and future work, we retain the original replicate labels (1, 3, and 5), which correspond to the designations of the respective base populations. Sex-limited selection on males was imposed through repeated crosses between ML males and clone-generator (CG) females (fig. S1a). CG females cause paternal transmission of the X chromosome to sons rather than daughters, supply a Y chromosome, and force co-segregation of the major autosomes (cII and cIII); we refer to this co-segregating X–II–III unit as a “target haplotype.” Competition among 1,050 target haplotypes for reproductive success with CG females each generation constituted one round of ML selection. Completely asexual propagation was avoided by allowing a small fraction (5%) of ML genomes to recombine as diploids in females (fig. S1b) in a “recombination compartment”. The selection experiment ran for more than 80 generations.

Experimental flies

We systematically investigated adaptation to the release of intralocus sexual conflict (IaSC), both with and without the features of the ML selection treatment, examining these features individually and in combination. The specific composition and production of test animals and competitors are described in full below. Briefly however, combined genetic effects were assessed in two “genetic backgrounds”: (i) clone-generator (CG), retaining all of the ML selection genetic context, or (ii) control-like (C) background lacking all these features. Individual components of the CG genetic background were tested in isolation to determine their independent contributions. The role of sexual co-evolution was examined using CG and control (C) females to evaluate male adaptation to an evolutionarily arrested versus a novel mating environment.

Test males

All test males assayed carried target haplotypes [cI(X), II and III] from ML / MC populations. To place target haplotypes in the CG background, we crossed males from each treatment to CG females, as per routine selection. Red-eyed sons from these crosses carry a haploid genome from the target genotype, combined with a CG derived (maternally inherited) Y chromosome, cytoplasm, and translocated autosomes (fig. S2a). The crosses conducted to produce males in the C background are described in detail in Thyagarajan *et al.* (2025), where a subset (C-background males competing for C females) of the fully factorial dataset presented here was included as a repeat assay to test the consistency of the observed lack of a male fitness

effect. Briefly, we use MC derived males to sire test offspring with either MC females (producing MC test animals) or females carrying ML-evolved chromosomes from the recombination compartment. Male offspring from these crosses carry a haploid genome from either a ML or MC selected population, combined with control derived cytoplasm, a (paternally-inherited) Y chromosome, and translocated autosomes (fig. S2b); and are distinguished by their wildtype phenotype.

Marked Competitors and Interacting Females

Phenotypically-marked males and females were necessary for assessing competitive ability of both males and females. We used lines backcrossed to have recessive eye colour markers (either “peach”, p^p , or recessive brown bw^l) in the control (MC) genetic background. Both competitor stocks were outbred and deemed vigorous; we refer to them collectively as “competitors” although the specific marker used is identified in each assay described below.

We took into consideration potential adaptation via sexual selection by assaying test males with both clone-generator (CG) females and control-derived females. Males were evaluated for competitive reproductive fitness, mating success, mating latency, copulation duration, fecundity induction of mates, sex-ratio drive, and sperm offense (P2), while mate harm was assessed using CG females only.

CG Genetic Components - Males

To explore specific components of the CG background, we combined target haploid genomes with individual CG-derived genetic elements while drawing the remaining genetic background from controls. These test groups—termed “CG-cyto,” “CG-Y” and “CG-auto”—carried a cytoplasm, autosome pair, or Y chromosome from the CG females, allowing us to isolate the effects of each component. For these assays, we measured only competitive reproductive fitness relative to a competitor population, focusing on the net consequences of each CG-derived genetic component.

(i) Cytoplasm. The cytoplasm of the CG female, including potentially foreign mitochondria, initially seemed to be inseparable from the compound X (DX) chromosomes because both are maternally inherited. However, we recognized that one way to acquire the cytotype of the CG females without the compound-X chromosomes was to isolate females that had undergone a breakdown of the DX chromosome; such a female, and her maternal descendants, could be crossed with males from another population to replace the nuclear genome. We began collecting these females, which occur at a very low frequency, however most were infertile and it took numerous attempts before we could establish a “DX-cyto” line. Using this fertile mutant female, we crossed in translocated autosomes (carrying a bw^D marker) and a balancer X chromosome ($FM7a$) to suppress recombination. Likewise, we also crossed the translocated autosomes and balancer X into the control MC cytotype, creating two cyto-lines

labelled FMTW-CG (carrying the CG cytotype) and FMTW-C (carrying the control MC cytotype).

Female FMTW flies were crossed to ML and MC males to capture haploid target genomes in female offspring. Daughters carrying these target haplotypes (balanced with FM and *bwD* translocated autosomes) were then crossed to MC males, to produce male offspring carrying the CG cytoplasm, with control Y and autosomes (fig. S3a). These “target” males were thus phenotypically wild-type, heterozygous for control autosomes, and with normal (paternal) inheritance of the Y chromosome.

(ii) CG-Y males. To produce males with maternal Y-inheritance, we first crossed MC males to dominantly-marked CG females. Sons from these crosses carry a CG-derived Y chromosome, along with control derived autosomes. In parallel, we crossed ML and MC males to FMTW-C females, producing daughters carrying a target (ML/MC) haploid genome (balanced with FM and *bwD* translocated autosomes). These sons and daughters respectively were crossed to produce target male offspring carrying the CG Y chromosome, with control autosomes and cytoplasm (fig. S3b).

(iii) CG-auto males. To produce males carrying the translocation (t2;3) from the clone generator system, we first backcrossed the CG males to MC females to create a line homozygous for CG autosomes. We then used crosses with MC males to replace the CG derived Y chromosomes while retaining the translocated autosomes. Males from this line were crossed to females homozygous for either ML or MC target haplotypes to produce target male offspring carrying the CG autosomes, with control (MC) cytoplasm and Y chromosomes (fig. S3c).

CG Genetic Components - Females

As with males, we expressed ML and MC haploid genomes in female flies, each combined with a single genetic element of the CG background at a time, to test for correlated responses to male adaptations. These females either carried a CG-derived cytoplasm with MC derived X and autosomes (CG-cyto), or CG translocated autosomes with an MC derived X and cytoplasm (CG-auto; and were tested for competitive reproductive fitness.

(i) CG-cyto. To capture the CG cytotype, we backcrossed MC males to the FMTW-CG line to replace the autosomes and one of the X chromosomes with MC derived chromosomes. In parallel, we crossed ML and MC males to dominantly-marked CG females. Sons from this cross were mated with the backcrossed females to produce target female offspring carrying the CG cytoplasm, heterozygous for control autosomes (fig. S4a).

(ii) CG-auto. To study the impact of CG autosomes upon females, we backcrossed MC males to CG females to produce males homozygous for the translocated autosomes, in combination with a MC derived Y chromosome. These males were then crossed to females

homozygous for ML or MC target haplotypes to produce target female offspring carrying the CG autosomes, control cytoplasm, and either ML or MC X-II-III haplotypes. (fig. S4b).

Assays

The design used for competitive reproductive fitness (CRF), mate choice and sperm competition assays are described in detail in Thyagarajan *et al.* (2025). They are described briefly below, with additional notes on any deviations from the original design made to accommodate the CG females.

Competitive Reproductive Fitness (CRF) assays

CRF was measured as competitive siring/damming success from mating arenas (ventilated $\frac{1}{2}$ pint bottles, with ~ 7 mL of food) containing 30 target animals combined with 30 marked competitors of the same sex and 50 individuals of the opposite sex. Reproductive success was measured by phenotyping the progeny using eye colour. In assays using CG females, we assessed 10 such competition arenas for both MLs and MCs within each replicate; and collected all the eggs laid (typically yielding 1-2 vials of ~ 80 viable individuals). For all other CRF assays, we assessed 6 such competition arenas and collected 6 vials of ~ 80 eggs. The fully factorial CRF assay across genetic background and female type was performed at generation 64, and the CG component male CRF assays were conducted over generations 68–72. CG-cyto and CG-auto female CRF was assayed in generation 83.

Mate Choice Assays

Female mate choice (or male-male competition) was assayed in generation 66 by combining a single virgin female (CG or competitor) with a target male and competitor male in vials with ~ 4 mL food. We identified the sire by phenotyping the progeny using eye colour. The resulting brood was also sexed and enumerated to test for fecundity induction (male influence on female egg output) and sex ratio. Matings were observed to estimate mating latency and copulation duration. For each treatment (within each replicate pair of lines), we assessed 50 such competition vials.

Sperm Offense Assays

Sperm offense (P2) was assayed (in generation 74) by first mating groups of virgin CG or competitor females with marker-matched competitor males in vials (12 female–male pairs per vial; ~ 4 mL food). Following removal of the competitor males under brief CO_2 anesthesia, females were held for 24 hours before being exposed to focal males (10 per vial). From each vial, 10 randomly selected females were then transferred to individual egg-laying tubes, yielding 10 P2 estimates per competition vial. P2 was scored as the proportion of offspring sired by the focal males, with 30 replicate competition vials scored per treatment within each replicate line pair.

Male Mate Harm and Fertility Assay

Mate harm was assayed (in generation 76) by measuring CG female mortality and productivity in response to being exposed to males. Following the crossing design, eggs of test animals were collected to produce densities of ~80 larvae per vial. Test males were collected on day 10-11. On day 12, 10 test males were combined in vials with 12 virgin CG females of the same age, in vials with ~4mL food. Flies were held together for days 12-14, at which point the males were removed from the females using gentle anaesthesia. Female mortality was recorded daily for 7 days starting with the introduction of males, and the productivity of the females post mating was measured in the form of pupal counts.

Statistical Analysis

Statistical analysis was conducted in R version 4.6.0 -- "Because it was There" (R Core Team 2026). Generalised linear mixed, beta-binomial, and Gaussian models were fitted using 'lme4' (Bates *et al.* 2015) and 'glmmTMB' (Brooks *et al.* 2017). Fixed-effect inference was based on 'car::Anova' (Fox & Weisberg 2019), corresponding to Wald χ^2 tests for generalised linear models and generalised linear mixed models, and F-tests for Gaussian linear models. Estimated marginal means were obtained using 'emmeans' (Lenth 2024), and planned contrasts using contrast (Kuhn *et al.* 2022).

For Gaussian models, residual normality and homogeneity were assessed using residual histograms, fitted diagnostic plots, and Shapiro–Wilk tests. For generalised linear and mixed models, residual dispersion, uniformity, and outlier structure were evaluated using simulated residual diagnostics implemented in DHARMA (Hartig 2024).

Where possible, analyses followed a common factorial structure including selection, (genetic) background, female type, and replicate as fixed factors, with all interaction terms included. Exceptions to this structure are noted for mate harm assays and isolated genetic element assays, where simplified factor structures were applied as described below. Across fitness, sperm competition, and mate harm assays, assay contest identity was included as a random effect to avoid pseudo-replication; a contest refers to an experimental grouping or trial in which multiple focal flies were assessed within a shared reproductive or survival assay context. Where significant interactions with selection were detected, planned contrasts between levels of selection regime were conducted within each condition using estimated marginal means. Adjusted significance thresholds were calculated using the Sidák correction to maintain comparison-wise $\alpha = 0.05$.

Competitive reproductive fitness (CRF) was analysed using binomial generalised linear mixed models with a logit link function. The response variable assessed here was the count of offspring of the focal fly versus that of competitors. Equivalent models were used for all isolated clone-generator genetic element assays, with selection regime and replicate population included as fixed factors and assay contest identity included as a random effect.

323 To study the mating success of target males in female mate choice trials, we used a
324 binomial generalised linear model with a logit link function, where trial outcome (success versus
325 failure for the focal male) served as the response variable. Mating latency, mating duration, and
326 fecundity induction were analysed using Gaussian linear models with the same fixed-effects
327 structure. Mating latency was log-transformed prior to analysis to improve residual normality.
328 Brood sex ratio was analysed using a binomial generalised linear model fitted to the number of
329 male and female offspring produced.

330 For sperm offense, the response variable was parameterised as competitor sperm defence
331 ($1 - P_2$), representing the probability that the first male successfully defended paternity against
332 the focal second male. The response variable was inverted because direct modelling of P_2
333 resulted in overdispersion. This inverted response was then analysed using a beta-binomial
334 generalised linear mixed model with a logit link function, to accommodate a zero-inflated
335 dataset.

336 Mate harm was analysed using a discrete-time survival framework. Female survivorship
337 across seven days (including male cohabitation phase and the post-removal phase) was modelled
338 using a binomial generalised linear mixed model fitted to interval-specific mortality probabilities
339 conditional on the number of females remaining alive at the beginning of each interval. As only
340 CG females were assayed in this experiment, female type was not included as a fixed factor. All
341 other factors and their interactions were included. Likewise, CG female productivity following
342 exposure to males was analysed using a Gaussian linear model with the same fixed effects.

RESULTS

Competitive Reproductive Fitness (CRF)

There was a strong adaptive response to selection in male CRF. This response was context dependent, varying with genetic background and sexual environment. Selection regime and genetic background both significantly affected CRF (both $p < 0.001$), with additional significant selection $\times$ background and female type $\times$ background interactions (both $p < 0.001$) (table S1.1; fig. 1). These interactions were explored using pairwise comparisons, which showed that ML males had significantly higher CRF than MC males in three of four test conditions (table S1.2). The magnitude of this improvement in focal male paternity depended on context, increasing with CG genetics and/or CG females: 66% in the CG background (29.5% vs 17.8%), 26% with CG females (46.8% vs 37.2%), and >100% when both were combined (22.4% vs 11.0%). In a C background with C females, the improvement was marginal (6%; 34.1% vs 32.2%).

The observed patterns suggest that selection favoured male-benefit variants within the ML populations, although their adaptation remained shaped by the specific conditions under which selection occurred, including adaptation to features of the selection treatment.

Mate Choice

ML males showed higher mating success and faster mating initiation, but shorter copulation duration compared with MC males, consistently across all tested conditions. Selection regime ($p < 0.001$), female type ($p = 0.013$), and replicate population ($p < 0.001$) significantly affected male success in mate choice trials (table S2.1). Overall, ML males achieved a mean mating success rate approximately 23% higher than MC males (57.4% vs 47.1%; fig. 2a); with advantages of 29.6%, 23.8%, and 11.8% in populations 1, 3, and 5, respectively.

Mating latency was affected by selection regime alone ($p = 0.048$), with no effects of female type, genetic background, replicate population, or their interactions (table S3). ML males initiated mating 18% faster than MC males (14.05 ± 2.08 min vs 17.17 ± 2.89 min; fig. 3a). Copulation duration was influenced by selection regime ($p = 0.008$), genetic background ($p = 0.007$), and female type ($p = 0.005$), with no significant interactions detected (table S4). Although ML males initiated mating more rapidly, they exhibited approximately 4% shorter copulations than MC males (19.76 ± 0.41 min vs 20.49 ± 0.43 min; fig. 3b). Together, these results provide evidence for male-benefit adaptations at the level of premating interactions. If copulation duration is taken as a proxy for sperm allocation or mating investment, the reduced duration in ML males suggests that post-copulatory investment was not a target of selection in this regime. Alternatively, reduced copulation duration may reflect differences in the intensity or structure of mating interactions, potentially arising as a correlated by-product of selection on mating interactions, particularly under conditions involving feeble CG females.

Fecundity Induction, Offspring Sex Ratio, Sperm Offense

Across all tested conditions, selection had no effect on the impact of male mating on female fecundity (fecundity induction), offspring sex ratio, or sperm offense (P2), suggesting that male-benefit adaptations were limited to premating traits.

Fecundity induction was influenced by genetic background, female type (both $p < 0.001$), replicate population ($p = 0.021$), and the female $\times$ background interaction ($p < 0.001$) (table S5). Selected males displayed marginally higher offspring counts than control males did (27.80 ± 2.20 vs 27.18 ± 2.46 ; fig. 3c). The effects of genetic background and female type are expected given that both CG females and CG genetic background result in high levels of aneuploidy due to the translocated autosomes and production of X-XX and Y0 eggs resulting from the compound X chromosome. This also pertains to corresponding effects observed for competitive reproductive fitness and P2, where they manifest in competitive reproductive outcomes rather than as direct measures of fecundity counts.

Mirroring fecundity induction, sperm offense was shaped by genetic background and female type, and their interaction (all $p < 0.001$) (table S7). ML males exhibited slightly higher P2 than MC males (84.8% vs 82.5%; fig. 2b). Offspring sex ratio was invariant across all factors (table S6), with ML males producing broods comprising 50.5% males vs 49.4% for MC males (fig. 3d).

Mate Harm

The survival trajectory of clone generator (CG) females (post exposure to males) showed effects of selection ($p = 0.003$, fig. 4a). Final female survivorship was 31–35% higher after exposure to the selected (ML) males compared with MC control males, depending on genetic background of the males; with or without CG genetics (CG: 48.1% vs 36.7%; C: 64.4% vs 47.8%). Productivity likewise depended on background: in the CG background there was no significant effect of selection (40.0 ± 5.36 vs 43.5 ± 4.76), whereas in the C background ML males produced approximately 53% higher productivity than MC males (104.0 ± 7.54 vs 68.0 ± 8.33 ; selection $p < 0.001$; fig. 4b). ML males thus appear to have evolved reduced mate harm, manifested in both improved immediate survival and productivity of their mates, at least under some test conditions.

Genetic Background Components

Since the fitness differences between ML and MC males were marginal when tested in entirely control-like conditions, we sought to parse out the individual effects of the different genetic manipulations employed. ML advantage in the presence of a given genetic element may reflect either compensatory adaptation to an imposed cost or epistatic coupling between the adaptive response and said genetic element. For flies carrying the foreign (CG) cytotype, ML males displayed a 18% gain in competitive reproductive fitness (36.0% vs 30.4%, $p = 0.017$; fig.

5b), with no replicate or interaction effects. In females with the CG cytotyp, CRF depended on replicate ($p = 0.01$) and the selection $\times$ replicate interaction ($p = 0.006$). Overall, we observe a recovery in ML female fitness (fig. 5e) with only replicate 5 remaining significantly lower in MLs relative to MCs ($\sim 9\%$; 51.6% vs 56.9%, $p = 0.005$, no effect in replicates 1 or 3).

In contrast, males with the translocated CG autosomes showed no effect of selection or interactions, with ML males exhibiting 29.7% competitive reproductive fitness versus 26.0% in MC males (fig. 5a). Correspondingly, female CRF with CG autosomes remained reduced under ML selection ($\sim 22\%$; 16.9% vs 21.5%; fig. 5d), driven by effects in replicate 3 (15.1% vs 19.6% $p < 0.001$) and 5 (15.4% vs 24.7%, $p < 0.001$), but not in replicate 1. Given that ML females exhibited lower fitness than MC females in the absence of CG autosomes, the persistence of this pattern in the presence of these autosomes suggests limited effects of the translocated autosomes.

For males carrying the CG Y chromosome, CRF was influenced by selection ($p < 0.001$), replicate ($p < 0.001$), and their interaction ($p = 0.002$), with an overall $\sim 33\%$ increase in ML males (37.6% vs 28.4%; fig. 5c). Effects were significant in replicates 1 (43.1% vs 31.2%, $p < 0.001$) and 3 (34.0% vs 19.8%, $p < 0.001$), but not in replicate 5.

Overall, we find that the CG cytoplasm and Y chromosome reveal context-dependent male-benefit effects of selection. The CG cytoplasm further shows a corresponding recovery in female fitness, suggesting sexually concordant benefits in this genetic context. By contrast, the translocated CG autosomes show no evidence of male adaptation and, correspondingly, no recovery in female fitness. Notably, while no replicate $\times$ selection interactions were detected for CRF, mating success, or sperm competition in the full CG genetic background, such effects emerge in these partitioned analyses. This suggests that similar adaptive outcomes may be reached through divergent evolutionary routes across replicate populations.

DISCUSSION

Selection experiments on sexual conflict in *D. melanogaster* based upon the male-limited (ML) selection protocol developed by Rice (1996, 1998) have been conducted on a single base population of fruit flies, LH_M (Rice 1998, Prasad *et al.* 2007), leaving us wondering about the generality of the earlier results. In addressing this, we recently reported that application of the ML selection protocol provided mixed evidence for the resolution of intralocus sexual conflict in three newly investigated populations of *D. melanogaster* (Thyagarajan *et al.* 2025). We found a clear signal of reduced fitness in females expressing male-limited (ML) evolved chromosomes, and males that exhibited increased fitness in some assays but not others, potentially reflecting adaptation to, or epistatic linkage with, unintended sources of selection. Unintended selection may arise from two aspects of the ML protocol: the evolutionarily arrested sexual-selection environment created by repeatedly introducing clone-generator (CG) females from a separate population as mates for the evolving males; and the unusual genetic background of the breeding design, including paternal X and maternal Y sex chromosomal inheritance by sons, marked and translocated autosomes, and a potentially novel cytoplasmic background. We devised a battery of experimental assays to dissect the evolutionary response to male-limited selection across conditions that systematically retain or remove the features of the ML selection protocol. We show that these populations exhibited a substantial response to selection, with generalized improvements consistent with release from intralocus sexual conflict, alongside context-dependent fitness effects shaped by male genetic background and female identity.

At the level of competitive reproductive fitness, the adaptive response was striking but context dependent. ML males were more than twice as fit as controls when assayed under the complete CG selection environment, with smaller but substantial fitness gains when either element of that environment was removed, culminating in only a marginal difference under fully control-like conditions. When partitioned into the relevant spheres of sexual selection, we found that the fitness increase in selected males was exclusively in the pre-copulatory realm; in the form increased competitive mating success and lower latencies to mating, with no significant changes between selected and control lines in post-copulatory effects, such as fecundity induced in mates, offspring sex ratio or sperm offense (P2). Importantly, these component traits were consistent across assay conditions, indicating that ML selection produced general improvements in pre-copulatory performance despite the context dependence of overall fitness.

These results align precisely with earlier work on LH_M-derived flies, which reported enhanced male fitness at a cost to female fitness (Prasad *et al.* 2007), acting through enhanced male attractiveness (Bedhomme *et al.* 2008), with no response in post-copulatory performance (Jiang *et al.* 2011); thereby extending this pattern to independently sourced populations, supporting the broader generality of these responses. In doing so, they are partially in contrast with Rice (1998), whose work implicated a response in the realm of sperm competition and ejaculate-female interactions in the evolved male fitness improvement. Given that sperm competition is already a male-limited trait, the absence of a post-copulatory improvement under

ML selection is perhaps not unexpected. The improved postcopulatory performance reported by Rice (1996, 1998) may instead reflect generalized fitness gains by males through differences in available standing genetic variation. This possibility is consistent with the fact that both the Prasad *et al.* suite of studies and the present work use long-domesticated populations, likely closer to evolutionary equilibrium in their laboratory environments relative to those used by Rice (1996, 1998). In this scenario, the latter may have retained greater opportunity for broad fitness gains due to greater standing genetic variation. If so, more recent work may more specifically reveal outcomes of IaSC resolution, with reduced contribution from broad, non-specific fitness gains associated with laboratory adaptation.

In addition to the improved sperm competition, Rice (1996) reported increased mate harm by ML males to CG females in the context of arrested intersexual coevolution. Since both increased mortality and female fecundity resulted from just a single mating, Rice inferred ejaculate-female interaction rather than sustained harassment as the causal agent. Here, we find consistency with the Rice's framework of male adaptation to static target females; but once again, the opposite with respect to the trait itself: when mating with the CG females, ML-selected males caused substantially *less* mate harm than controls, in both genetic backgrounds. Early in the selection experiment, CG females were so frail that we often had difficulty maintaining the ML populations at full size in the face of their rapid mortality. Since we did not detect the kinds of fertility-inducing effects or advantages in sperm competition that have been suggested to drive male ejaculate toxicity in this species, our results raise an intriguing question about the nature of intersexual interactions: can selection favour reduced mate harm in the absence of monogamy or kin selection? It appears that less harming males can be favoured by selection, at least when male fitness is directly constrained by female survival; as facilitated by fragile females and an interaction sustained over a two-day period - as in the conditions we maintained our lines. More broadly, altered mating-rate conflict can shift the evolutionary balance between male reproductive success and female costs, as demonstrated in experimental evolution studies manipulating interlocus sexual conflict (Holland & Rice 1999; Nandy *et al.* 2013). The reduced mate harm also leads us to retrospectively speculate about postcopulatory performance. Selection to minimise harm toward CG females may have come at the expense of sperm defense (P1), potentially reflecting a correlation where ejaculate-mediated and interaction-based harm—traits that would normally reduce female remating and thereby enhance P1—are instead selected against under these conditions. In fact, such a reduced P1 against non-CG females would neatly reconcile our findings of increased attractiveness with the marginal CRF gain under totally control-like assay conditions.

Returning to the role of the genetic background in the fitness response, we sought to further dissect the contribution of its individual genetic components. Like previous users of this system, we originally assumed that the Y chromosome was inert and could not be modified by maternal transmission. However, in our previous study (Thyagarajan *et al.* 2025), the principal difference

between assays that detected large versus marginal gains in male fitness was the presence of the CG-derived Y chromosome, implicating it as the primary suspect in mediating the observed context dependence. Here, we confirm this effect: the CG Y chromosome alone conferred a clear reproductive advantage to ML males relative to controls, even in an otherwise control-like genetic background and in competition for control females. Two, not mutually exclusive, explanations may account for this response. First, the Y chromosome forms part of the static genetic background imposed by the CG system. Like the foreign cytoplasm and translocated autosomes discussed below, its repeated association with evolving haplotypes could favour adaptation to its specific allelic composition. Second, since the CG Y chromosome spends half of its evolutionary history in females, selection during female expression or even carriage through the female germ line may produce unpredictable selection outcomes, favouring compensatory adaptation by ML males to a potentially "feminized" Y chromosome.

In addition to Y chromosomes, our results suggest that the CG cytoplasmic background is also relevant to male fitness. This cytoplasm, and the mitochondria it contains, can be traced matrilineally to the origin of the compound-X chromosome in the 1940s (Muller 1943), raising the possibility of substantial divergence from the mitochondria present in the populations used in our experiments. Consistent with this speculation, recent sequencing shows that CG females carry a mitochondrial genome resembling the OreR/w¹¹¹⁸ lineage markedly distinct from that of the Ives-derived stocks used herein (F. Camus, pers. comm.). ML males experienced this cytotype throughout selection, whereas controls encountered it only when experimentally tested. Having directly excluded infection by *Wolbachia* using PCR assays (data not shown), adaptation to foreign mitochondria remains the most plausible source of the cytoplasmic effects we found. Such a response is consistent with compensatory evolution of male function following mother's curse effects due to the nuclear-mitochondrial mismatch. Here, any reduction in male fitness would have been transient and likely confined to the early generations of selection, well before our first assays at generation 48. A similar mismatch may underlie the early-generation fitness decline reported in male-limited X-chromosome selection experiments (Abbott *et al.* 2013), although the mitochondrial genotype of the LH_m base population relative to OreR/w¹¹¹⁸ remains unknown.

Previously, we showed that female fitness of selected haplotypes declined substantively following male limited selection (Thyagarajan *et al.* 2025). Here we ask if male adaptation to the genetic features takes advantage of sex-limited selection to improve males through routes that are indifferent (sex-limited), or antagonistic, rather than beneficial to female fitness. When the CG cytotype was isolated and tested, we found a recovery of fitness in females expressing the selected haplotypes relative to control females, suggesting that the nuclear response to the foreign cytotype produces sexually concordant benefits. If we assume that mother's curse effects would favour male-limited compensatory evolution due to its exclusive costs for male function (although this assumption need not necessarily hold), our results are more consistent with broad nuclear-mitochondrial coadaptation following cytoplasmic mismatch, rather than a strictly male-

specific compensatory response to mother's curse. By contrast, we detected no comparable compensatory response to the translocated autosomes: these elements did not significantly improve evolved male performance, nor did they alter female fitness. The contrasting effects of these CG-derived components are notable, particularly given that the smaller genomic elements appear to have stronger effects than the much larger autosomal component. One possible explanation is that the cytoplasm and Y chromosome impose stronger selection due to their potential for mismatch and unusual inheritance respectively, whereas the translocated autosomes may represent a large element with less change in allelic content.

Further, component specific fitness effects were not entirely consistent across replicate populations, with variation in which mechanisms allowed fitness to improve in the CG genetic background. For example, unlike replicates one and three, selected males in population five show little improvement over controls under two component test conditions (the CG cytoplasm and Y chromosome); female results concur. The strongest fitness gain for replicate five is seen in performance with the translocated (t2;3) autosomes of the clone generator (CG) system; selected males in replicate five show a trend of improved fitness relative to their paired controls. Taken together, the 3 selected populations appear to follow somewhat different routes in adapting to the unusual features of the selection protocol, potentially reflecting differences in the standing genetic variation available to selection.

The adaptation to elements of the selection protocol displayed in our selection experiment raises concerns about previous experimental work conducted using the clone generator system. CG flies have been widely used as dams in breeding designs to conduct hemiclinal analyses (Chippindale *et al.* 2001; Gibson *et al.* 2002; Byrne & Rice 2005; Lew & Rice 2005; Lew *et al.* 2005; Linder & Rice 2005; Pischedda & Chippindale 2006; Long & Rice 2007; McKean *et al.* 2008; Rode & Morrow 2009; Innocenti & Morrow 2010; Mallet *et al.* 2011; Tennant *et al.* 2014; Hill *et al.* 2017; Ruzicka *et al.* 2019) and male-limited selection experiments (Rice 1996; Prasad *et al.* 2007). In different iterations, authors have taken various levels of precaution against possible artefacts, ranging from Rice (1998) where everything but the CG Y chromosome was controlled for, to Rice (1996) where none of these artefacts were controlled for. A common theme running through all these studies is the use of test animals with the novel cytoplasm and potentially "feminized" Y chromosomes derived from the CG females. We find that both can introduce fitness effects, differentially affecting selected and control animals in selection experiments, and potentially distorting trait variance in hemiclinal analyses. It is important to note that most of these experiments (all but McKean *et al.* 2008, Mallet *et al.* 2011 & Tennant *et al.* 2014) use the LHM base population, which may respond differently to selection pressures described herein.

Our work highlights both the strengths and pitfalls of experimental evolution using genetic manipulations. On the one hand, we see striking evidence for rapid and substantial adaptation – to sex, genetic background, and to mates – but, on the other the potential for artefactual impacts, and thus unintended sources of evolution that can easily be misinterpreted. Our own paper

highlights how technically challenging to follow these studies can be, even for veteran experimentalists. Furthermore, long term domestication can lead to changes in the nature of genetic variation in populations and may influence the outcomes of similar selection experiments conducted on the same population at different time points (e.g., Rice 1996 vs. Jiang *et al.* 2011). Quantifying intralocus sexual conflict is also inherently difficult because the sexes experience different forms of competition and necessarily interact with one another in reproduction. Our own experiments attempted to establish a common currency with assays we refer to as Competitive Reproductive Fitness (CRF), drawing upon the long-term domestication of the source populations and our ability to create marked replica versions of the Control populations as competitors. This allowed us to nest focal test animals within a close approximation of the ancestral populations and to thereby gauge relative fitness changes; still our CRF assays make assumptions about the nature of competition within each sex (e.g., for resources in females and for mates in males) which may or may not be fully justified. Intralocus sexual conflict therefore continues to be a challenging phenomenon to investigate, even in model systems in the laboratory, where these simplified systems nonetheless reveal rich adaptive landscapes.

REFERENCES

- Abbott, J. K., Bedhomme, S., & Chippindale, A. K. (2010). Sexual conflict in wing size and shape in *Drosophila melanogaster*. *J. Evol Biol*, 23(9), 1989–1997.
- Abbott, J.K., Innocenti, P., Chippindale, A.K. & Morrow, E.H. (2013). Epigenetics and Sex-Specific Fitness: An Experimental Test Using Male-Limited Evolution in *Drosophila melanogaster*. *PLOS ONE*, 8, e70493.
- Abbott, J.K., Chippindale, A.K., Morrow, E.H. (2020). The microevolutionary response to male-limited X-chromosome evolution in *Drosophila melanogaster* reflects macroevolutionary patterns. *J Evol Biol*, 33: 738–750.
- Ågren, J. A., Munasinghe, M., & Clark, A. G. (2019). Sexual conflict through mother's curse and father's curse. *Theoretical population biology*, 129, 9–17.
- Bates, D., Maechler, M., Bolker, B. & Walker, S. (2015). Fitting Linear Mixed-Effects Models Using lme4. *Journal of Statistical Software*, 67, 1–48.
- Bedhomme, S., Prasad, N.G., Jiang, P.P. & Chippindale, A.K. (2008). Reproductive Behaviour Evolves Rapidly When Intralocus Sexual Conflict Is Removed. *PLOS ONE*, 3, e2187.
- Bonduriansky, R. & Chenoweth, S.F. (2009). Intralocus sexual conflict. *Trends in Ecology & Evolution*, 24, 280–288.
- Branco, A. T., Brito, R. M., & Lemos, B. (2017). Sex-specific adaptation and genomic responses to Y chromosome presence in female reproductive and neural tissues. *Proceedings. Biological sciences*, 284(1869), 20172062.
- Brooks, M.E., Kristensen, K., van Benthem, K.J., Magnusson, A., Berg, C.W., Nielsen, A., Skaug, H.J., Maechler, M. & Bolker, B.M. (2017). glmmTMB Balances Speed and Flexibility Among Packages for Zero-inflated Generalized Linear Mixed Modeling. *The R Journal*, 9, 378–400.
- Byrne, P. G., & Rice, W. R. (2005). Remating in *Drosophila melanogaster*: an examination of the trading-up and intrinsic male-quality hypotheses. *J Evol Biol*, 18(5), 1324–1331.
- Chippindale, A.K., Gibson, J.R. & Rice, W.R. (2001). Negative genetic correlation for adult fitness between sexes reveals ontogenetic conflict in *Drosophila*. *Proceedings of the National Academy of Sciences*, 98, 1671.
- Collet, J. M., Fuentes, S., Hesketh, J., Hill, M. S., Innocenti, P., Morrow, E. H., ... & Reuter, M. (2016). Rapid evolution of the intersexual genetic correlation for fitness in *Drosophila melanogaster*. *Evolution*, 70(4), 781-795.

- 644 Connallon, T. & Clark, A.G. (2014). Balancing selection in species with separate sexes:
645 insights from Fisher's geometric model. *Genetics*, 197, 991–1006.
- 646 Connallon, T. & Knowles, L.L. (2005). Intergenomic conflict revealed by patterns of sex-
647 biased gene expression. *Trends in Genetics*, 21, 495–499.
- 648 Connallon, T. & Matthews, G. (2019). Cross-sex genetic correlations for fitness and fitness
649 components: Connecting theoretical predictions to empirical patterns. *Evolution*
650 *Letters*, 3, 254–262.
- 651 Cosmides, L. M., & Tooby, J. (1981). Cytoplasmic inheritance and intragenomic conflict.
652 *Journal of theoretical biology*, 89(1), 83–129.
- 653 Dapper, A. L., Diegel, A. E., & Wade, M. J. (2023). Relative rates of evolution of male-
654 beneficial nuclear compensatory mutations and male-harming Mother's Curse
655 mitochondrial alleles. *Evolution*, 77(9), 1945–1955.
- 656 Dowling, D.K., Friberg, U., Lindell, J., 2008. Evolutionary implications of non-neutral
657 mitochondrial genetic variation. *Trends Ecol. Evol.*, 23, 546–554.
- 658 Frank, S., & Hurst, L. (1996). Mitochondria and male disease. *Nature* 383, 224
- 659 Gemmell, N. J., Metcalf, V. J., & Allendorf, F. W. (2004). Mother's curse: the effect of
660 mtDNA on individual fitness and population viability. *Trends in ecology &*
661 *evolution*, 19(5), 238–244.
- 662 Gibson, J.R., Chippindale, A.K. & Rice, W.R. (2002). The X chromosome is a hot spot for
663 sexually antagonistic fitness variation. *Proceedings of the Royal Society of London.*
664 *Series B: Biological Sciences*, 269, 499–505.
- 665 Hartig, F. (2024). DHARMa: Residual Diagnostics for Hierarchical (Multi-Level / Mixed)
666 Regression Models. R package version 0.4.7.
- 667 Holland, B. & Rice, W.R. (1999). Experimental removal of sexual selection reverses
668 intersexual antagonistic coevolution and removes a reproductive load. *PNAS*, 96(9),
669 5083–5088.
- 670 Hill, M.S., Ruzicka, F., Fuentes, S., Collet, J.M., Morrow, E.H., Fowler, K. *et al.* (2017).
671 Sexual antagonism exerts evolutionarily persistent genomic constraints on sexual
672 differentiation in *Drosophila melanogaster*. *bioRxiv*, 117176.
- 673 Flinham, E., Savolainen, V., Otto, S.P., Reuter, M. & Mullan, C. (2025). The maintenance of
674 genetic polymorphism underlying sexually antagonistic traits. *Evolution Letters*, 9,
675 259–272.
- 676 Fox, J. & Weisberg, S. (2019). *An R Companion to Applied Regression*, Third edition. Sage,
677 Thousand Oaks CA.

- 678 Frank, S. A., & Hurst, L. D. (1996). Mitochondria and male disease. *Nature*, 383(6597), 224.
- 679 Innocenti, P. & Morrow, E.H. (2010). The Sexually Antagonistic Genes of *Drosophila*
680 *melanogaster*. *PLOS Biology*, 8, e1000335.
- 681 Jiang, P. P., Hartl, D. L., & Lemos, B. (2010). Y not a dead end: epistatic interactions
682 between Y-linked regulatory polymorphisms and genetic background affect global
683 gene expression in *Drosophila melanogaster*. *Genetics*, 186(1), 109–118.
- 684 Jiang, P. P., Bedhomme, S., Prasad, N. G., & Chippindale, A.K. (2011). Sperm competition
685 and mate harm unresponsive to male-limited selection in *Drosophila*: an evolving
686 genetic architecture under domestication. *Evolution; international journal of organic*
687 *evolution*, 65(9), 2448–2460.
- 688 Keaney, T. A., Wong, H. W. S., Dowling, D. K., Jones, T. M., & Holman, L. (2020).
689 Mother's curse and indirect genetic effects: Do males matter to mitochondrial genome
690 evolution? *Journal of evolutionary biology*, 33(2), 189–201.
- 691 Kidwell, J.F., Clegg, M.T., Stewart, F.M. & Prout, T. (1977). Regions of stable equilibria for
692 models of differential selection in the two sexes under random mating. *Genetics*, 85,
693 171-183.
- 694 Kuhn, M., Weston, S., Wing, J., Forester, J. & Thaler, T. (2022). *contrast: A Collection of*
695 *Contrast Methods*. R package version 0.24.2.
- 696 Lande, R. (1980). Sexual dimorphism, sexual selection, and adaptation in polygenic
697 characters. *Evolution*, 34, 292–305.
- 698 Lemos, B., Araripe, L. O., & Hartl, D. L. (2008). Polymorphic Y chromosomes harbor
699 cryptic variation with manifold functional consequences. *Science (New York,*
700 *N.Y.)*, 319(5859), 91–93.
- 701 Lew, T.A. & Rice, W.R. (2005) Natural selection favours harmful male *Drosophila*
702 *melanogaster* that reduce the survival of females *Evol Ecol Res* 7: 633-641
- 703 Linder, J. E. & Rice, W. R. (2005). Natural selection and genetic variation for female
704 resistance to harm from males. *Journal of evolutionary biology*, 18(3), 568–575.
- 705 Long, T.A.F. & Rice, W.R. (2007). Adult locomotory activity mediates intralocus sexual
706 conflict in a laboratory-adapted population of *Drosophila melanogaster*. *Proceedings*
707 *of the Royal Society B: Biological Sciences*, 274, 3105-3112.
- 708 Mallet, M.A. & Chippindale, A.K. (2011). Inbreeding reveals stronger net selection on
709 *Drosophila melanogaster* males: implications for mutation load and the fitness of
710 sexual females. *Heredity*, 106, 994-1002.
- 711 McKean, K.A., Yourth, C.P., Lazzaro, B.P. *et al.* (2008). The evolutionary costs of
712 immunological maintenance and deployment. *BMC Evol Biol* 8:76.

- 713 Muller, H. J. (1943). A stable double X chromosome. *Drosophila Information Service*, 17,
714 61-62.
- 715 Nandy, B., Gupta, V., Sen, S., Udaykumar, N., Samant, M.A., Ali, S.Z. & Prasad, N.G.
716 (2013). Evolution of mate-harm, longevity and behaviour in male fruit flies subjected
717 to different levels of interlocus conflict. *BMC Evolutionary Biology*, 13, 212.
- 718 Pischedda, A. & Chippindale, A.K. (2006). Intralocus Sexual Conflict Diminishes the
719 Benefits of Sexual Selection. *PLOS Biology*, 4, e356.
- 720 Poissant, J., Wilson, A.J. & Coltman, D.W. (2010). Sex-specific genetic variance and the
721 evolution of sexual dimorphism: a systematic review of cross-sex genetic correlations.
722 *Evolution*, 64, 97–107.
- 723 Prasad, N.G., Bedhomme, S., Day, T. & Chippindale, A.K. (2007). An Evolutionary Cost of
724 Separate Genders Revealed by Male-Limited Evolution. *The American Naturalist*,
725 169, 29-37.
- 726 R Core Team. (2026). R: A language and environment for statistical computing (Version
727 4.6.0)
- 728 Rand, D. M., Haney, R. A., & Fry, A. J. (2004). Cytonuclear coevolution: the genomics of
729 cooperation. *Trends in ecology & evolution*, 19(12), 645–653.
- 730 Rice, W.R. (1996). Sexually antagonistic male adaptation triggered by experimental arrest of
731 female evolution. *Nature*, 381, 232-234.
- 732 Rice, W.R. (1998). Male fitness increases when females are eliminated from gene pool:
733 Implications for the Y chromosome. *Proc. Natl. Acad. Sci.*, 95, 6217-6221.
- 734 Rode, N.O., and Morrow, E.H. (2009), An examination of genetic variation and selection on
735 condition in *Drosophila melanogaster* males. *Entomologia Experimentalis et*
736 *Applicata*, 131, 167-177.
- 737 Ruzicka, F., Hill, M.S., Pennell, T.M., Flis, I., Ingleby, F.C., Mott, R. *et al.* (2019). Genome-
738 wide sexually antagonistic variants reveal long-standing constraints on sexual
739 dimorphism in fruit flies. *PLOS Biology*, 17, e3000244.
- 740 Thyagarajan, H., Sayyed, I., Baroodi, M. G., Kowal, J. A., Day, T., & Chippindale, A. K.
741 (2025). Mixed evidence for intralocus sexual conflict from male-limited selection in
742 *Drosophila melanogaster*. *Journal of Heredity*, esaf072.
- 743 Wright, A.E., Fumagalli, M., Cooney, C.R., Bloch, N.I., Vieira, F.G., Buechel, S.D. *et al.*
744 (2018). Male-biased gene expression resolves sexual conflict through the evolution of
745 sex-specific genetic architecture. *Evolution Letters*, 2, 52–61.
746

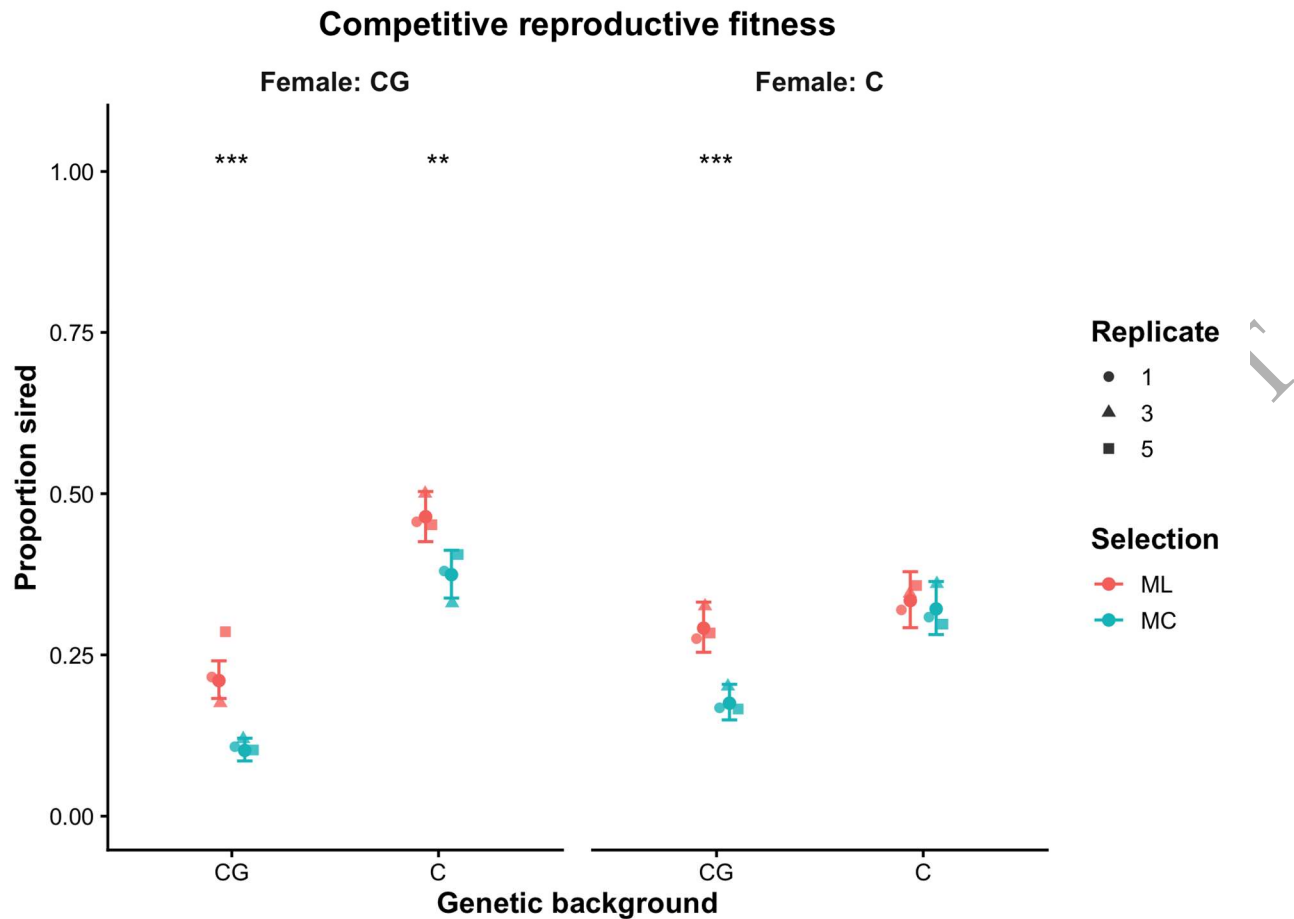


Figure 1.

The competitive reproductive fitness of ML-selected males was greater than control (MC) males in each of four different female-by-genetic background combinations, however the relative magnitude varied considerably. “CG” refers to both the clone-generator females themselves, used to create male-limited selection, and to the genetic alterations they transmit to their sons (a t2;3 translocation, maternal Y chromosome, and foreign cytoplasm). Fitness was measured as mating success relative to marked control-type males. Adaptation by ML males appears to have occurred in both interacting with the CG females and in functioning with the unusual genetics they inherit from CG dams; with neither factor present, the fitness advantage of 6% was nonsignificant.

Alt text caption: Two-panel point-and-error plot of competitive reproductive fitness, measured as the proportion of offspring sired. The left panel shows matings with CG females and the right panel shows matings with control (C) females. The x-axis in each panel shows genetic background (CG or C), and the y-axis shows the proportion of offspring sired, ranging from 0 to 1. Male-limited selection (ML; orange) has higher mean reproductive fitness than control males (MC; blue) in all four female-by-genetic-background combinations, although the magnitude of

the difference varies among combinations. The largest differences occur when CG females are used, whereas the difference is smallest when both C females and the C genetic background are present. Error bars show confidence intervals, and jittered shapes represent replicate population means.

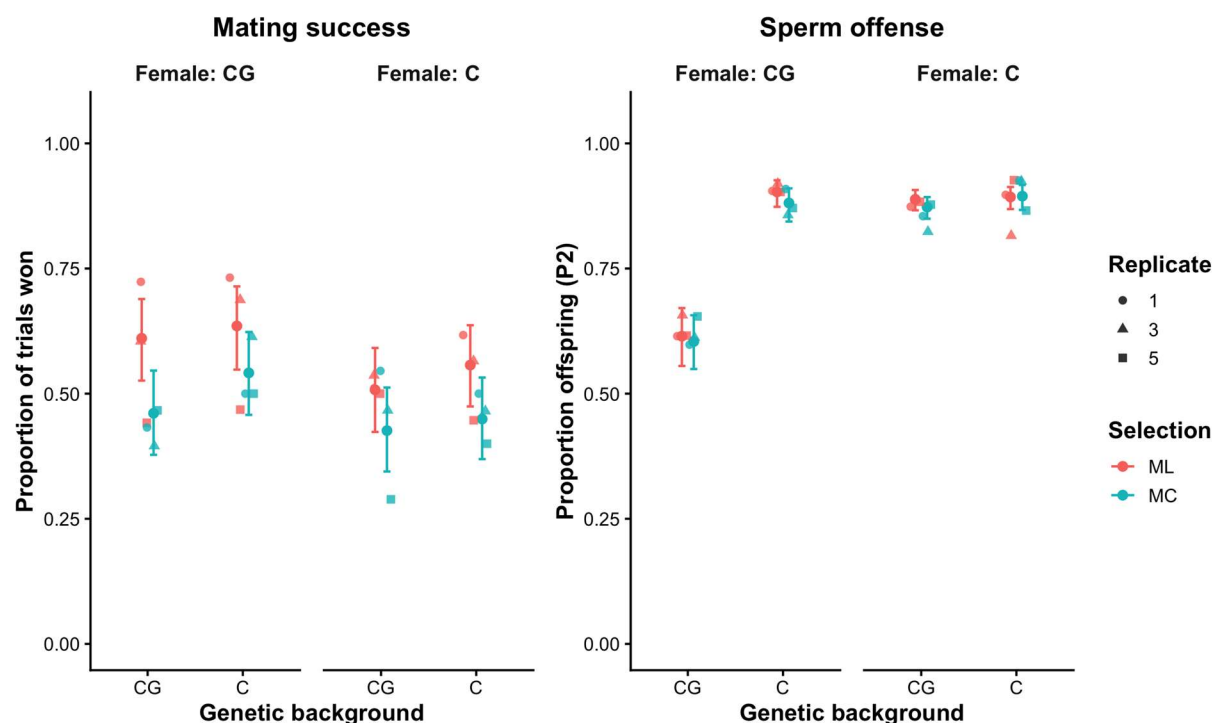


Figure 2.

Pre- and post-copulatory reproductive success of males in relation to female type (Clone Generator or Control) and genetic background (including features related to CG mothers or Control). (a) Mating success, measured as frequency of mating relative to marked control-type males, was about 23% higher in selected compared to control males ($p < 0.001$) across all four treatments and showed little evidence of interaction between the different factors. (b) Sperm offense, or P2, measures the fertilization success of the last male to mate with a female. No differences between selected and control males were detected in this post-copulatory fitness component.

Alt text caption: Two sets of two-panel point-and-error-bar plots showing components of reproductive fitness for ML (orange) and MC (blue) males. The left set shows mating success and the right set shows sperm offense. Within each set, the left panel shows CG females and the right panel shows control (C) females. The x-axis shows genetic background (CG or C), and the y-axis shows the proportion of trials won for mating success or the proportion of offspring (P2) for sperm offense. ML males generally have higher mating success than MC males, across genetic background and female type. In contrast, ML and MC males show broadly similar sperm offense across female and genetic-background combinations, with values generally higher for the

C than the CG genetic background. Error bars show confidence intervals, and jittered shapes represent replicate means.

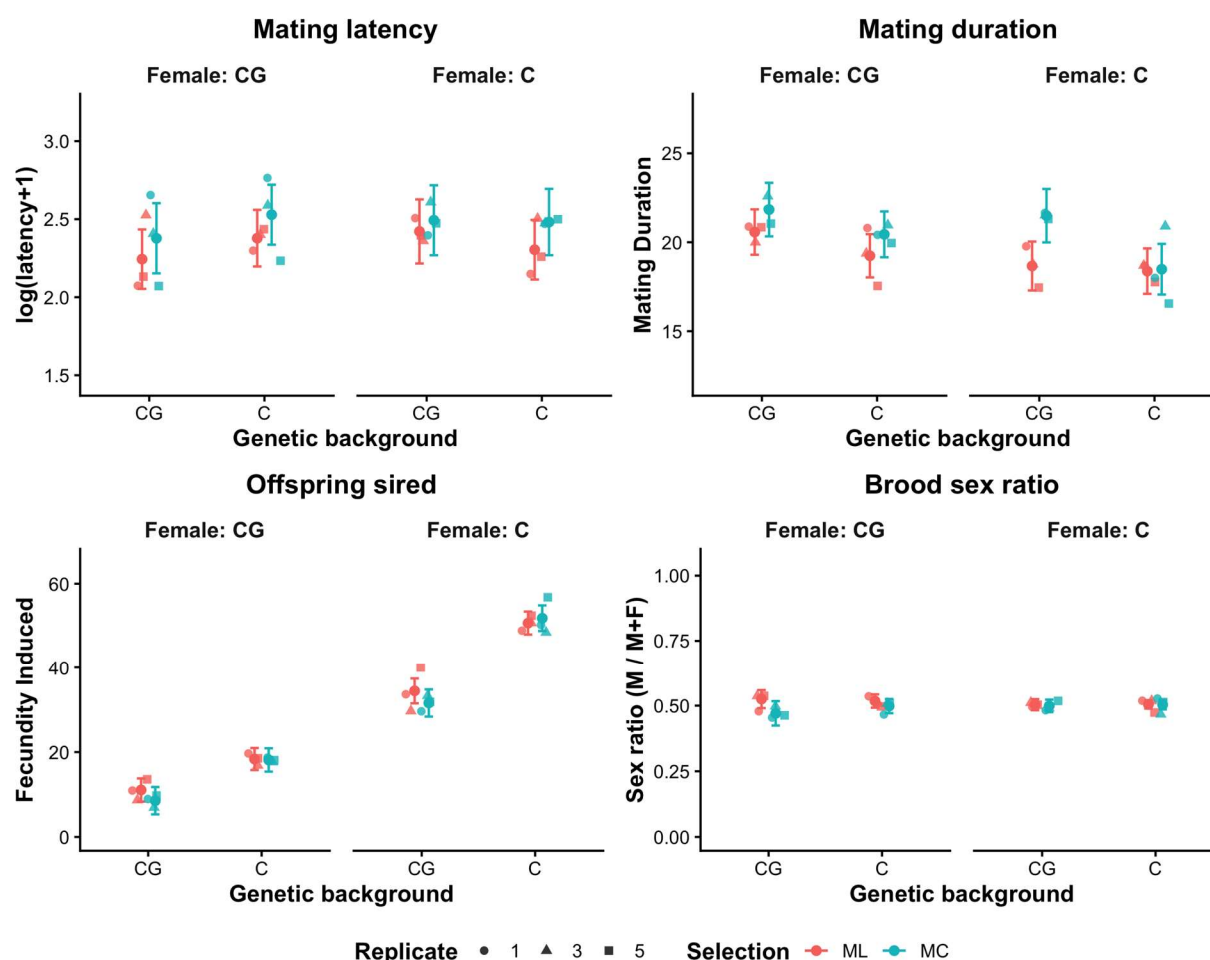


Figure 3.

(a) Selected (ML) males acquired matings more rapidly with both kinds of females (clone generator or control) and with haplotypes expressed in both genetic backgrounds (derived from CG or control dams) compared with control (MC) males, suggesting increased attractiveness that was independent of female type. The speed differential of approximately 18% was significant ($p = 0.048$) and insensitive to interactions. (b) The duration of mating was slightly (4%) but significantly ($p = 0.008$) reduced in selected (ML) compared to control (MC) males, consistently across female type and genetic background. (c) Female egg production in relation to male mate (fecundity induction) did not significantly differ between selected and control males, but the clone-generator genetics significantly reduced both innate female fecundity and the fecundity induction ability of males. (d) No departures from a 1:1 sex ratio were found, despite the hypothetical advantage a meiotically-driving X chromosome would have in the selected populations.

Alt text caption: Four two-panel point-and-error plots arranged in two rows, depicting mating latency, mating duration (in the top row), fecundity induction, and brood sex ratio (in the bottom row) respectively in the y axis. Each panel compares ML (orange) and MC (blue) males across female type (panel) and genetic background (x-axis). Error bars show confidence intervals and jittered shapes represent replicate means. (a) ML males acquire matings more rapidly than MC males across female types and genetic backgrounds, indicating consistently greater mating speed. (b) ML males have slightly shorter mating durations than MC males, with a similar pattern across female types and genetic backgrounds. (c) Female fecundity and fecundity induction do not differ substantially between ML and MC males, but CG genetics and females are associated with lower female fecundity. (d) Sex ratios remain close to 1:1 across treatments, with no evident difference associated with male selection or genetic background.

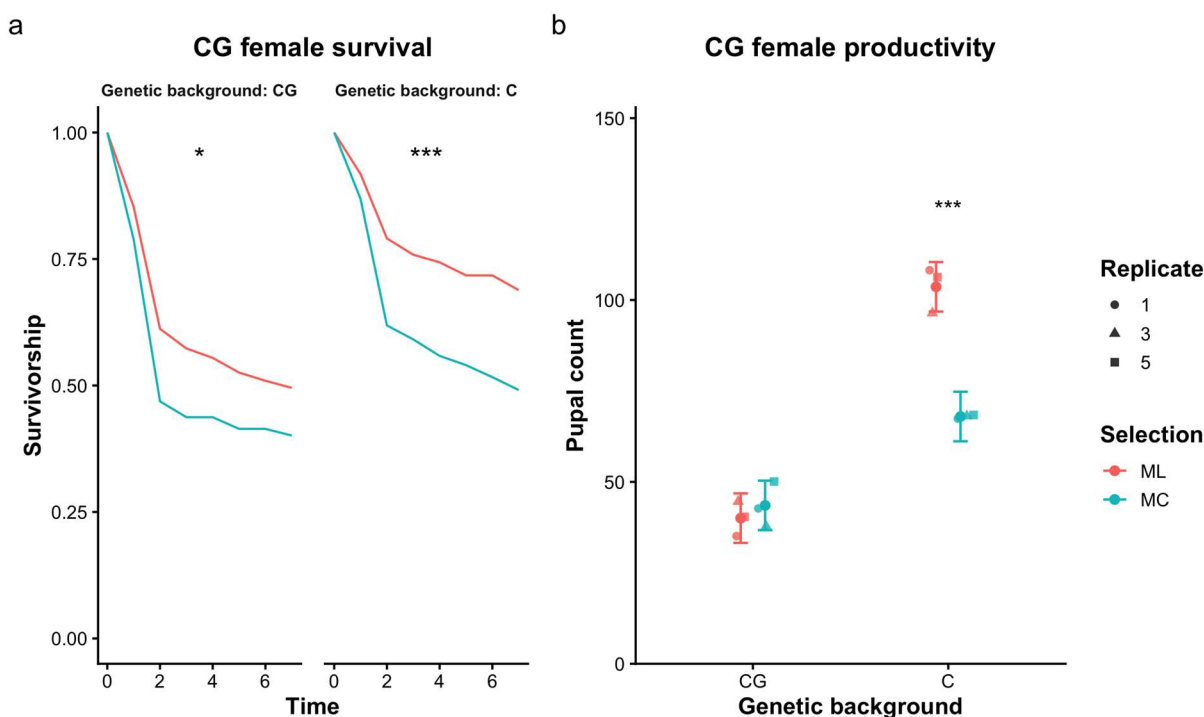


Figure 4.

(a) Survival of clone generator (CG) females over six days after exposure to selected (ML) or control (MC) males. Males were tested with both the CG (t2;3; cytotype; reversed sex chromosome inheritance) and control genetic backgrounds; in both cases, the survival of females was significantly greater after exposure to ML males ($p = 0.003$). (b) The difference in productivity (offspring pupal count) between male genetic backgrounds is expected due to aneuploidies resulting from the translocated autosomes in the CG males. Productivity of the CG females was significantly greater ($p < 0.001$) after exposure to ML males, but only in the control male genetic treatment. Altogether these results suggest lower levels of mate harm by selected males.

Alt text caption: Two plots comparing female responses to exposure to ML (orange) and MC (blue) males across genetic backgrounds. Plot (a), on the left, represents CG female survivorship upon exposure to males in the y-axis over time in days (x-axis), across male genetic backgrounds (panel). Plot (b), on the right, depicts CG female productivity post exposure, measured as offspring pupal count, across male genetic backgrounds (x-axis). For plot (b) error bars show confidence intervals and symbols represent replicate means. (a) Female survivorship is higher following exposure to ML than MC males across both genetic backgrounds. (b) Female productivity is higher following exposure to ML than MC males in the control genetic background, but does not differ substantially between male types in the CG genetic background. Overall, the plots indicate reduced mate harm to CG females from exposure to ML males.

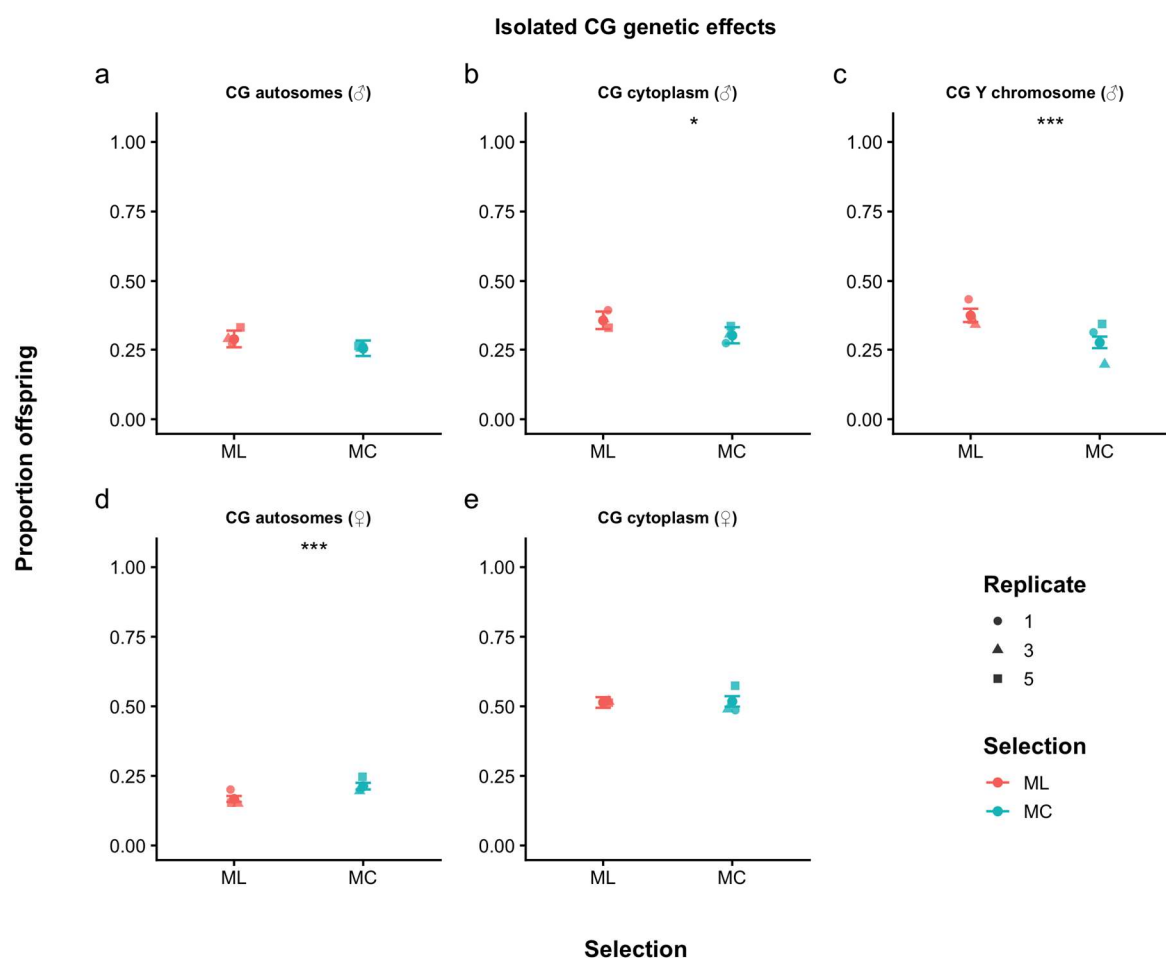


Figure 5.

In each assay, competitive reproductive fitness was measured with all remaining components of the genetic background held in the control state. (a, d) In the presence of the translocated autosomes, fitness patterns were qualitatively similar to those expressed in control-like conditions, with female fitness remaining lower for ML than MC genotypes ($p < 0.001$) and no significant difference in male fitness. (b, e) There was evidence of adaptation to the foreign

cytoplasm supplied by CG dams, with ML males outperforming MC males ($p < 0.05$) and ML female fitness recovering to levels comparable with MC females. (c) Selected males also performed better than controls in the presence of the CG-derived Y chromosome, which may have been modified by selection during female expression or by maternal transmission.

Alt text caption: Five point-and-error plots arranged in three panels across the top row and two panels across the bottom row, showing competitive reproductive fitness when individual components of the CG genetic background are isolated while all other genetic components are held in the control state. Top row plots represent male fitness, bottom row female fitness assays. The x-axis shows selection treatment (also represented through colour: ML - orange; MC - blue), and the y-axis shows proportion of offspring. Error bars show confidence intervals, and jittered symbols represent replicate means; circles, triangles, and squares distinguish replicates. (a) CG-associated translocated autosomes in males: male fitness is similar between ML and MC genotypes, with no evident difference between selection treatments. (b) CG-associated cytoplasm in males: ML males have higher reproductive fitness than MC males. (c) CG-derived Y chromosome in males: ML males have higher reproductive fitness than MC males. (d) CG-associated translocated autosomes in females: female fitness is lower for ML than MC genotypes. (e) CG-associated cytoplasm in females: female fitness is similar between ML and MC genotypes, indicating recovery of fitness in ML females.