

**“FLY AROUND & FIND OUT” - AN ANTHOLOGY OF SELECTION
EXPERIMENTS ON SEXUAL CONFLICT AND SPECIATION IN *DROSOPHILA*
*MELANOGASTER***

by

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ABSTRACT

Sexual conflict is a catchment area, collecting all forms of fitness costs from disharmony between the sexes of a species. In chapter 1, I introduce the forms of sexual conflict, and chart a history of the field by reviewing selection experiments used to study this conflict through manipulations of mating systems, reproductive outcomes, and genetic inheritance patterns. In addition to synthesizing this discipline, I present open questions as identified by theory, and under-studied but ecologically and taxonomically valuable research directions.

The genetic material of the sexes is shared but undergoes divergent selection pressures. Amongst other outcomes, this is expected to contribute to the maintenance of heritable trait polymorphisms. The existence and genomic coordinates of these sexually antagonistic (SA) polymorphisms represent important open questions in evolutionary biology. Chapter 2 describes a selection experiment designed to address these questions. Divergent selection on the sexes is artificially replaced with directional (male-limited “ML”) selection, which is expected to result in male-benefit adaptive changes and a reduction of heritable variance at SA loci. Replicating test conditions used in previous ML experiments, I find evidence for improved male fitness at the cost of female fitness and critically, a reduction of genetic variance for male fitness. However, I use an improved breeding design to demonstrate that these benefits are not general, suggesting that the selection design includes alternative sources of selection that obscure our ability to characterize SA loci and variance. Chapter 3 addresses this concern, identifies sources of alternative selection, and critiques this system of experimentation. I demonstrate adaptive responses to a nuclear-cytoplasmic mismatch (due to male-harming cytoplasmic genes under matrilinear inheritance), a shared Y chromosome (as against the usual patrilinear Y) and arrested male-female coevolution.

In chapter 4, I deviate from the central theme established thus far to study speciation. Populations with restricted gene flow and varied selection pressures are expected to undergo reproductive isolation (RI) over evolutionary timescales. Using a long-term evolution experiment (LTEE) with divergent life-history selection between allopatric populations, I demonstrate the evolution of bi-directional premating RI through female mate choice tempered by asymmetric mating rate conflict in the populations.

CO-AUTHORSHIP

Chapter 4 has been published with co-authors, as follows:

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Author contributions: CER and HT contributed equally to this work and are joint lead authors. CER, HT, and AKC developed the overall theme of the study; HT and AKC designed the experiments; CER, HT & AKC carried out the experiments; CER and HT analysed the data; CER and HT wrote the first draft of the paper; CER, HT, and AKC contributed to the final draft and revisions.

Chapter 1, 2, & 3 (or sections thereof) are / will be submitted for publication with co-authors as follows:

1. Thyagarajan, H., Day, T., & Chippindale, A.K. What have 3 decades of experimental evolution studies on sexual conflict taught us?

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My place of work is situated on stolen land. Today, this territory is included in the Dish with One Spoon Wampum Belt Covenant, an agreement between Haudenosaunee and Anishinaabe nations to peaceably share and take care of the resources of the Great Lakes. I enter as a newcomer in the spirit of friendship and respect, but with the knowledge that the structures and institutions I access are built on colonization and genocide.

Second, I work in a land far from home, in search of improved economies and the research infrastructures they make possible. These improved economies themselves are a product of unfettered access to imperialist accumulation by the European powers of the 19th and 20th centuries, followed by the enforced terms of the neoliberal consensus that continues to fail the median global citizen while enriching the owners of our economy. It is in this historical context that this country scapegoats international students and workers (as of 2024), limiting their access to these resources in the name of fairness - a wretched distortion of logic, facilitated by an appeal to “common sense”.

Within an Indian context, the limited access to these resources is far from evenly distributed. As a member of an oppressor caste, I have had disproportionate access to an education and additional resources; through an accident of birth and the endogamous networks of power that come with it.

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I'd like to add a little time capsule here, taken from remarks made at my exit seminar: Previous graduating students in this department have created a beautiful culture of leaving us with advice from their lived experience on the way out. Here is my daisy for the chain: The funding system that we are dealing with is neither fair nor sustainable. Even with the department's efforts to stretch a tight budget, and supervisor grants running dry to cover us, graduate workers have had to endure shamefully poor financial health¹. In our unfunded years (which everyone seems to go through) this is especially harrowing, as we have had no chance to build savings to weather the storm unless we take on additional jobs at the cost of our research and mental health. We need to stand together to demand better from this university & province. At the end of the day, a university is a public institution. It is a disservice to all of us to understand it as a business first, and an insult to mismanage that business with such austerity. On that note, I would like to omit this university and its administration from these here acknowledgements.

¹ At the time of writing, the standard funding package for my position is ~26k CAD/yr. Tuition and ancillary fees costs ~8k/yr. The average room for rent in this city costs ~9k/yr, a single bedroom apartment ~18k/yr. Groceries, utilities, car, phone and insurance bills are enough to put us in the red.

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LIST OF ABBREVIATIONS

IeSC: Inter-locus sexual conflict
IaSC: Intra-locus sexual conflict
SA: Sexual antagonism / sexually antagonistic
 $r_{g,m-f}$: Intersex genetic correlation
SSDR: Sex specific dominance reversal
MCN: Middle class neighbourhood
ML: Male limited
MC: Matched control
ML-X: Male limited X chromosome
FL-X: Female limited X chromosome
CG: Clone generator
SD: Sexual dimorphism
ART: Alternative reproductive tactic
SBGE: Sex biased gene expression
RI: Reproductive isolation
LMC: Local mate competition
ID: Inbreeding depression
LH: *Drosophila melanogaster* stock named after Larry Harshman
IV: *D. melanogaster* stock named after Bruce Ives
JB: *D. melanogaster* stock named after Amitabh Joshi
LH_M: “Moderate” density stocks derived from LHs
CO: “Control old” stocks derived from IVs
ACO: “Accelerated” stocks derived from COs
FEJ: “Fast eclosing” stocks derived from JBs
RNA: Ribonucleic acid
DNA: Deoxyribonucleic acid
CRF: Competitive reproductive fitness
RC: Recombination chamber
PO: Post oviposition
Cr: Recessive competitor
P2: Sperm offense
ML_{DD}: ML double dose
ML_{SD}: ML single dose
ML_{SD(a)}: ML single dose, exclusively autosomal
LTEE: Long term evolution experiment

CHAPTER 1: GENERAL INTRODUCTION, LITERATURE REVIEW

What have 3 decades of experimental evolution studies on sexual conflict taught us?

INTRODUCTION

The sexes are anisogamous - they produce very different gamete types: large, energetically costly, and limited; and small, inexpensive, and abundant. The downstream energetic economics of these two strategies frequently lead to distinct selection pressures, influencing the physical phenotype, behaviour, life history, reproduction, and ecology (Parker *et al.* 1972). These divergent selection pressures can result in fitness costs for sexually reproducing organisms, both from direct agonistic interactions and indirect failures to optimize shared traits.

A widespread consequence of anisogamy is conflict over mating rate. Individuals producing cheap gametes should benefit at a steeper gradient with increased matings compared to individuals producing rate-limiting gametes (Bateman 1948). This asymmetry, taken in combination with relative levels of parental investment (Trivers 1972), mate availability measured in the form of operational sex ratios (Emlen & Oring 1977) and the variation in overall potential reproductive rate (Clutton-Brock & Vincent 1991) is expected to determine intensity of sexual selection acting upon each sex. While plenty of variations have been discovered around this theme (reviewed in Janicke *et al.* 2016a), the (perhaps overly) simplistic canon for promiscuous, internally fertilizing gonochorists is as follows: Females prioritize quality over quantity in mates, seeking traits indicative of male-specific attractiveness (Weatherhead & Robertson 1979), good genes (Hamilton & Zuk 1982), capacity to withstand handicaps (Zahavi 1975) or direct nuptial gifts (Zeh & Smith 1985). Conversely, males, with their reduced investment in offspring, tend to pursue a higher mating rate to maximize reproductive success (Clutton-Brock & Parker 1992). This difference can

lead to conflict where males attempt to mate with multiple partners while females are selective, potentially resulting in competition, coercion, mate harming or mate guarding behaviours (reviewed in Andersson, 1994; Simmons, 2001). A more nuanced treatment of the evolution of divergent mating incentives can be found in Parker (2014)'s "sexual cascade". Here, Parker synthesizes theory and multiple lines of evidence to present a plausible evolutionary timetable (along with the necessary parameters) for the evolution of behaviourally complex species with sexual selection and conflict, starting from ancestral isogamous eukaryotes.

These mating incentives can result in two forms of fitness load – (1) the sexes are forced to mate "sub-optimally" (*sensu* Holland & Rice 1998), i.e. deviate from their optimal mating rate and (2) the nature of these sexual interactions can induce physical (or chemical) harm (Parker 1979). Generally, this type of conflict is mediated by different traits / genes in females and males, e.g., chemical receptors and chemical signals, some likely sex-limited; and is therefore known as inter-locus sexual conflict (IeSC). IeSC is typically characterized by rapid cycles of "arms race" coevolution where males and females adapt to best their partner through novel adaptations. The poster example of this arms race is seen in water striders in the form rapid armament adaptation in aid of both male coercion and female resistance (Arnqvist & Rowe 2002). Given its overlap with sexual selection (which also facilitates co-evolutionary dynamics) the presence of IeSC is thought to be reliably detected through mate-harm (Rowe & Day 2006).

While the sexes may experience divergent selection pressures from sources outlined above, the majority of traits are expressed conjointly by males and females (i.e., strong positive intersex genetic correlations, $r_{g,m-f}$), potentially restricting them from achieving the

fitness optima of either sex (Parker 1979, Lande 1980). This scenario leads to a fitness load² at loci subject to divergent selection, where trait values are displaced from the fitness optima of both sexes by “tug-of-war” dynamics. Such conflict, mediated by genotype-by-sex interactions for fitness associated with loci of interest, is termed intra-locus sexual conflict (IaSC). Sexually antagonistic (SA) loci were originally predicted to exist by models of within-locus conflict (Owen 1953, Bodmer 1965, Karlin 1972; reviewed in Gavrillets 2014, Geeta Arun 2022). More recent theory suggests that conditions for IaSC are far more permissive than previously expected, with sexual antagonism emerging from differences in directional selection *or* phenotypic effects of mutations between the sexes (Connallon & Clark 2013). A prominent example of IaSC is observed in the fruit fly (*Drosophila melanogaster*) – partially clonal families exhibit opposite fitness effects when expressed as males and females, resulting in a negative intersex genetic correlation for fitness ($r_{w,g,m-f}$) (Chippindale *et al.* 2001). This fitness load is believed to be widespread due to the prevalence of divergent selection pressures and positive genetic trait correlations.

Indeed, it is likely that all existing sexual dimorphism has evolved, at least in part, to resolve IaSC. Potential routes to resolution include the evolution of sex-limitation through sex-specific dominance reversals (SSDRs) (Kidwell *et al.* 1977), gene transfer to sex chromosomes (Charlesworth & Charlesworth 1980), sex specific gene expression / splice variants (Rice 1984, cf. Connallon *et al.* 2010), genomic imprinting (Day & Bonduriansky 2004), or sex-specific condition dependence (Bonduriansky & Rowe 2005); and brood sex ratio adjustment on the basis of mate quality (Calsbeek & Sinervo 2004) (reviewed in Chenoweth *et al.* 2008, Stewart *et al.* 2010, Pennell & Morrow 2013).

² We advocate here to relabel this fitness load (perhaps to IaSC load). Previously, it has been referred to as ‘gender’ load rather than sex load, to distinguish it from the 2-fold cost of sex. However, this remains completely unrelated to the social construct of human gender, and causes harm when weaponized in errant rhetoric by reactionary bigots.

An important debate in this literature is the location of SA variance in the genome. Invasion conditions for SA alleles on sex limited chromosomes (Y/W) are easily predicted – as a default consequence of sex limitation, these see no sexual antagonism. Novel sex determining regions are expected to be enriched / linked with SA alleles that benefit the sex it is expressed in. However, as a consequence of complete recombination shutdown, these chromosomes are expected to degenerate over time. The haplodiploid X (or Z) however, presents a potential hotspot for SA variation. Specifically, recessive variation that is exposed by default in the haploid sex, and potentially masked in the diploid sex. Theoretical support for this idea is equivocal. Assuming no sex-specific dominance variance, Rice (1984) predicts an enrichment on the X chromosome, unlike Kidwell *et al.* (1977) who demonstrates that SSDRs would facilitate similar invasion conditions on autosomes (cf. Fry 2010). While we see evidence in support of Rice’s prediction (discussed in Ruzicka & Connallon 2020), we also see growing evidence supporting Kidwell *et al.*’s assumptions (reviewed in Grieshop *et al.* 2024).

Additionally, through divergent selection and strong genetic correlations, IaSC offers a clear mechanistic route to the maintenance of balanced polymorphisms (Prout 2000). This prediction has proved challenging to test empirically, but we discuss some evidence herein.

This would be an appropriate place to explain the choice to focus on the experimental evolution literature in response to these problems. However, given that the promise and pitfalls of this genre of protocols have been addressed adequately elsewhere (Prasad & Joshi 2003, Kawecki *et al.* 2012), we merely note that the volume of recent output in this area warrants synthesis and summary.

IaSC EVOLUTION EXPERIMENTS

Designing evolution experiments to study IaSC represents a fundamental challenge. Ideally, the downstream contributions of IaSC are tested in its absence - under conditions where selection acts upon the sexes concordantly, eliminating the genotype-by-sex interaction for fitness. In itself, such a system hardly needs an evolution experiment – asexual organisms are common, and even parthenogens that have recently transitioned from sexual to asexual reproduction are taxonomically abundant. However, the challenge here is the confound of recombination. In an abrupt switch from sexual to asexual reproduction, organisms also completely lose their ability to shuffle alleles and disrupt linkage groups, severely impacting their evolutionary trajectories for reasons unrelated to IaSC.

Three strategies have been applied to study IaSC: sex-limited selection using modified middle-class neighbourhood (MCN) designs, sex-limited selection using cytogenetic breeding designs, and selection on traits for changes in intersex genetic correlations ($r_{g,m-f}$). Selection experiments focussed on putative sexually antagonistic traits have also revealed evidence for IaSC, which we include in a section creatively named ‘trait selection’.

Middle-class neighbourhoods

In the classical MCN design, selection in the form of adult reproductive fitness is nullified by artificially ensuring that all individuals have equal reproductive output. Normally, this nullification of selection is used to study effects relating to mutation accumulation (Shabalina *et al.* 1997). Radwan *et al.* (2004) modified this design to create a female-limited MCN with bulb mites (*Rhizoglyphus robini*), to ask if good genes sexual selection on males indirectly benefits females in the form of a reduction in deleterious mutation load across both sexes. Here they equalized the reproductive fitness of all females,

while allowing male reproductive fitness to scale with male success under sexual selection in the target population. Additionally, they restricted both sexes to an MCN in a second treatment for comparison, along with multiple true controls with no restriction on reproductive fitness in either sex. Morrow *et al.* (2008) take this one step further with the explicit interest of establishing the existence of IaSC in fruit flies (*D melanogaster*). They not only set up female-limited MCNs *a la* Radwan *et al.*, but also introduce male-limited MCNs – where single males are allowed to mate with multiple (grouped) females, while keeping the number of offspring from each group of females constant. More recently, Martinossi-Allibert *et al.* (2019) (also see Baur & Berger 2020) describe a female-limited MCN analogous to the systems of Radwan *et al.*, but designed to test the role of sexual selection in seed beetles (*Callosobruchus maculatus*) adapting to novel environments. Rather than comparing their female-limited MCN to an MCN that nullifies selection in both sexes, they use a monogamy treatment that prevents sexual selection, while still allowing fecundity selection. Additionally, they reconstitute genetic variance in their baseline population by mixing individuals derived from isofemale lines, followed by 16 generations of equilibration under generic maintenance conditions. While Radwan *et al.* & Martinossi-Allibert *et al.* predict that selection acting on males might rescue both sexes from deleterious mutation load, Morrow *et al.* predict that the sexually antagonistic loci can undergo selection against deleterious mutations in one sex, with negative correlated changes in fitness effects for the other.

Radwan *et al.* found no differences in male and female fitness between the MCNs with and without sexual selection on males. Female fitness in both MCN populations of mites declined relative to true controls. In the absence of a fitness effect on males under sexual selection, they were unable to assess their initial hypothesis of good genes. Contrastingly, Martinossi-Allibert *et al.* find clear evidence for the good genes hypothesis in *Callosobruchus*, suggesting that on balance the genetic variance for fitness in this population

is sexually concordant. Morrow *et al.* found that the fitness of both sexes declined relative to controls in both treatments, and that the fitness of a sex declines more sharply when it is held in the MCN. This decline in fitness was primarily attributed to increased rates of mutation accumulation relative to controls (due to Muller's ratchet in small populations), with the deleterious effects of the mutations having positive genetic correlations between the sexes. Here, the greater decline in the sex withheld from selection is potential evidence for sexual antagonism – with the caveat that a similar outcome can be obtained from a preponderance of loci with sex-limited expression. Overall, we find equivocal results from these experiments. Where selection produces a response, we see one population displaying signatures of IaSC, while the other displays sexually concordant variation. We note that it is possible that the sexually concordant genetic variance is a result of insufficient equilibration post-panmixis. For instance, if certain isofemale lines were fortuitously preadapted to maintenance conditions, the newly mixed population will display temporary positive intersex genetic correlations for fitness (i.e. sexually concordant variance) until the selective sweep culminates in an equilibrated population.

Cytogenetic breeding designs

A second approach to sex-limited selection comes from *D. melanogaster* cytogenetic breeding designs. Shortly after the formalization of early IaSC theory, Rice (1992) developed an artificial sex-determining region by introgressing a pair of dominant autosomal eye-colour markers and manually culling all males that expressed them. The markers and linked regions could only be expressed and passed on by females, allowing selection to act in favour of the female fitness function alone, potentially fixing female-benefit male-detriment alleles at SA loci in the sex-determining region.

In a tour de force of experimental breeding design, Rice (1996, 1998) then created a system to passage entire haploid genomes through male-limited (ML) selection. Here, they capitalized on the lack of chiasma formation in *Drosophila* male gametogenesis (male gametes are not shuffled through molecular recombination); and utilized dams carrying marked translocated autosomes to prevent independent assortment of autosomes, and a compound X chromosome pair (XXY karyotype) to reverse the inheritance pattern of sex chromosomes. This system has since been used by Prasad *et al.* (2007) (also see: Bedhomme *et al.* (2008, 2011), Abbott *et al.* (2010), Jiang *et al.* (2011)), Thyagarajan *et al.*^a (chapter 2); and partially in Abbott *et al.* (2013, 2020), to create a male-limited X chromosome. Independently, this breeding design was foundational in creating the system of hemiclinal analysis (Chippindale *et al.* 2001, reviewed in Abbott & Morrow 2010, cf. Thyagarajan *et al.*^b (chapter 3)) and directed inbreeding for mutation accumulation designs (Mallet *et al.* 2011).

Lund-Hansen *et al.* (2020) reverse the direction of selection, using a recessively marked balancer X chromosome to create a female-limited X chromosome by manually culling males not expressing the marker phenotype (also see: Manat *et al.* (2021), Lund-Hansen *et al.* (2022)).

In all of these experiments, selected haplotypes are expected undergo directional selection, influenced exclusively by the fitness function of the sex that they are selected in. As a proof of principle, we expect fitness an increase when selected haplotypes are expressed in the sex that underwent selection, and decrease in the opposite sex. Selection is expected to act upon shared traits, with sex limited traits undergoing no direct changes in selection. However, sex-limited traits may still trigger IaSC through pleiotropic interactions with traits shared between the sexes.

Female limited selection

Rice (1992) found that their artificial female sex-determining region substantially reduced fitness when expressed in males, but did not produce a significant increase in female fitness. One possible explanation for such an outcome is simply relaxation of selection in males in the selected region, resulting in ‘silent’ male harming variation accumulating through mutations and drift. However, this is unlikely to have caused fitness reductions as large as 50% within 29 generations. Moreover, while female fitness did not increase, the population sex ratio grew significantly female biased over the course experiment. If brood sex ratios were modulated by the selected region, it is possible that female fitness improved within the selection experiment through a driving phenomenon, which would not be identified in a fitness assay. Additionally, given that Rice (1992) sex-limits a previously autosomal region, this study provides insights into theoretical expectations around novel sex determining loci and neo-sex chromosome formation, in the form of rapid accumulation of sexually antagonistic effects in linked regions shortly after it begins segregating. Rice (1992) does not comment on phenotypic changes, or component pieces of reproductive fitness.

Employing female-limited selection on the X chromosome through a different protocol, Lund-Hansen *et al.* (2020) found no change in female fecundity or male reproductive fitness for individuals carrying the female-limited X (FL-X) after 72 generations of selection. However, in their observations on shared traits, they find development time and thorax size to be consistently ‘feminized’ in both males and females, with selected haplotypes increasing size and reducing development time. This lack of response in fitness could be due to females already being close to their fitness optimum with respect to variation segregating on the X chromosome. For instance, an enrichment of dominant female-benefit variation on the X chromosome (due to its haplo-diploid nature) would create the necessary conditions for such patterns. In fact, given that recessive male-benefit variation can only undergo

selection under heterozygous condition by design, it is possible that variation on the balancer chromosome can consistently shield these alleles from selection throughout the selection experiment. However, they also note that this result may be artefactual – deriving from adaptations to the balancer chromosome and fitness costs specific to that genetic background, or the benign conditions of sexual selection / IeSC fostered by the feeble mutant males the females are exposed to. The change in phenotypic traits without mediated changes in fitness under generic test conditions suggests a role for artefacts, but the consistent feminization of both body size and development time suggests a possible role for the relaxation of sexually antagonistic selection at the X chromosome.

In a follow-up interested in correlated changes, Manat *et al.* (2021) use the selection experiment to study male reproductive fitness components in the form of pre- and post-copulatory success. Here, they identify a significant increase in sperm defense by males carrying the FL-X, and no significant differences in mating success (under no-choice conditions) and sperm offense. While non-significant, they find a nominal uptick in mating success and a decline in sperm offense in males carrying the FL-X chromosome. Together, these results could even point to sexually concordant outcomes to FL-X selection. However, these experiments are carried out not on individuals with introgressed Xs, but males carrying autosomes and Y-chromosomes subject co-evolving within the FL-X treatment – introducing potential sources of artefact in the results.

Male limited selection

In Rice (1996), we find the first evidence of fitness increasing in the sex allowed to experience selection. However, the core interest of this study is the interruption of intersex co-evolution between the selected males and the methodological “clone-generator” (CG) females. Since the CG females are derived from a separate population (exclusively to serve as

dams in the selected population) there is an interest in the male adaptations to “arrested” females. With this in mind, Rice describes an increase in multiple male fitness components for selected haplotypes relative to controls – in net fitness, remating rate and sperm defense; but in the very specific context of being tested under the selected conditions (i.e., in the heterokaryotypic state against CG females). Beyond adaptations specific to the CG females, these results also include adaptations to the various artefacts of the CG genetic background, including a novel (CG) cytoplasm, translocated autosomes, reversed sex chromosome inheritance and a shared (as against sex-limited) Y chromosome. For these reasons, this study does not allow us to make definitive conclusions about IaSC.

Fortunately, Rice (1998_a) returns to the question of IaSC to test male fitness of male-limited (ML) haplotypes under much more generic conditions. Here, using females from the baseline populations (carrying recessive markers) and an improved breeding design to produce test animals carrying no artefact in their genetic background (barring the Y chromosome), Rice once again demonstrates improved male fitness measures for selected haplotypes relative to controls – in overall fitness, mating latency, remating rate and sperm offense. This improvement in sperm offense, an already male-limited trait is especially interesting, as it suggests that there are perhaps pleiotropic sources of constraint preventing males from accessing their fitness optima. Sperm defense was not different between selected and control haplotypes, in contrast to Rice (1996). Much like Rice (1996), this study found selected males to be more harmful to their mates, inducing greater levels of mortality post mating. While fitness in the selected sex had clearly improved, Rice (1998_a) does not explicitly test female fitness, instead assaying retardation of development time as a proxy trait. Here, delayed development of females is interpreted as evidence for a reduction in female fitness, but could just as well point to masculinization of the phenotype under male-limited selection.

It is not until Prasad *et al.* (2007) that this is explicitly tested. Using an identical ML selection experiment design, Prasad *et al.* explicitly test fitness outcomes in males in and females, and demonstrate improved in fitness in the selected (male) sex at the cost of fitness in the other! However, it must be noted that the breeding design used here does not match the one used by Rice (1998_a). Here, test animals carry translocated autosomes³ in addition to the shared Y chromosomes (in males alone), exposing the assay to artefactual effects. In addition to this, male fitness is tested using CG females (*a la* Rice (1996)), allowing for female arrest effects to confound the findings. Beyond their analysis of fitness though, they show clear phenotypic evidence of masculinization with both reductions in body size and increases in development time for selected haplotypes relative to controls in both sexes. In a follow-up, Abbott *et al.* (2010) demonstrate clear masculinization of wing morphology, wing loading and allometry in both sexes. Not only do they find evidence of masculinization, they also demonstrate greater developmental stability for selected haplotypes in males compared to females measured through fluctuating asymmetry, suggesting that being ontogenetically more male-like is disruptive to development in females.

Bedhomme *et al.* (2008) extend this work to study behavioural responses to selection, demonstrating that male courtship behaviours reduce in selected animals, even as they continue to acquire the same number of matings as controls – indicating more attractive males. In contrast, females are found to be less attractive, and display reduced yeast-feeding behaviour, a critical component of female fecundity. Here, the test males do not include translocated autosomes, but carry the CG cytoplasm and the shared Y chromosome. While the contributions of artefactual adaptations to male behaviours are unclear, the changes in female behaviours are independent of such artefactual contributions, suggesting a clear

³ In a discussion from further work on the same selection experiment, Jiang *et al.* (2011) mention that unpublished data from Prasad *et al.* shows no artefactual effect of the autosomal translocations, but the breeding design used is not reported.

impact of the selection program on fitness components in females. Using the same design to produce test males, Jiang *et al.* (2011) investigate this selection experiment for changes in male post-copulatory traits - sperm competition and mate harm. Here, they find no differences between selected and control haplotypes, tested against ancestral and CG females, in sharp contrast to Rice (1996, 1998_a). Typically, there would be reason to be cautious in interpreting these results due to potential adaptations to the artefacts (*a la* Bedhomme (2008)), but in the absence of differences, it appears that these traits did not undergo any adaptation during the experiment. The parsimonious interpretation here is that sex-limited traits undergo no further changes during further sex-limited selection, but the authors also speculate about this non-response being a result of a lack of heritable variation for the traits in question, or as a consequence of complex male-by-male-by-female interactions. Likewise, a study on cuticular hydrocarbons (likely targets of sex-specific selection) revealed no impact of ML selection, which the authors argued could be due to resolved conflict at these loci (Bedhomme *et al.* 2011).

Abbott *et al.* (2013) revisit this system partially, constraining a population of X chromosomes to evolve in males, without restricting autosomes. Here too, they identify evolved differences in male fitness, with males carrying the male-limited X (ML-X) displaying higher reproductive fitness than controls. However, they demonstrate a second valuable finding using a control that undergoes a single generation of male limited X chromosome passaging. These animals, carrying the CG cytoplasm and shared Y chromosome (like ML-X males, but without a history of adaptation), displayed lower fitness than the controls, suggesting that ML-X adaptation is at least partially compensatory adaptation to fitness costs imposed by these artefacts. In a follow up, Abbott *et al.* (2020) showed that improved male fitness had not evolved at the cost of female fitness, with ML-X females displaying roughly the same fitness as controls. This, taken with the Lund-Hansen *et*

al. (2020)'s FL-X results, suggests that sex limited selection on the X chromosome does not reveal the enrichment of sexually antagonistic variation predicted by Rice (1984).

Additionally, Abbott *et al.* demonstrate a masculinization of locomotory activity in females, although the same phenotype was not significantly different from controls in males. Lastly, they found the ML-X transcriptome to be enriched for down-regulated genes in response to selection.

Most recently, Thyagarajan *et al.*^a (chapter 3) return to the ML system to study role of IaSC in the maintenance of genetic variance. Using a breeding design derived from Rice (1998_a) (avoiding all genetic artefacts barring the shared Y), and a hemiclonal analysis of genetic variance for fitness, this study reports a significant threefold reduction in genetic variance for fitness in males, and a significant increase in male fitness for the ML selected haplotypes. However, using an improved breeding design to test fitness without the shared Y chromosome, no fitness differences were found between the selected and control haplotypes when expressed as males. Female fitness for the selected haplotypes declined significantly relative to controls. In a follow-up Thyagarajan *et al.*^b (chapter 3) find evidence for local adaptation to the genetic background and arrested CG females, obscuring capacity to study the effects of IaSC through the ML selection experiment. Within the genetic background, significant compensatory adaptations are identified in response to the CG cytoplasm and shared Y chromosome, but not in response to the translocated autosomes. In congruence with Bedhomme *et al.* and Jiang *et al.*, this study finds that the improvement of selected male fitness under these conditions is mediated through mating success and reduced mate harm, with no significant differences observed in sperm offense, fecundity induction or sex chromosome drive. Further, Thyagarajan *et al.*^c (in prep) find partial masculinization of the phenotype – while development time of ML haplotypes increases in both sexes as expected, body size of ML haplotypes also increase relative to controls in both sexes. Here it must be

noted that an important difference in this evolution experiment is the shift to population cage maintenance, unlike the vial maintenance employed by Rice (1996) and Prasad *et al.* (2007). A more complex physical environment of selection may also alter the patterns of sexual selection acting upon traits like body size these populations (Yun *et al.* 2017, Malek & Long 2019).

Overall, these studies seem to consistently find that phenotypic traits evolve as predicted by sex limitation, providing strong evidence for sexually antagonistic genetic variance. A majority of these studies describe a similar agreement with theory in their findings about reproductive fitness, but there is need for caution here, as compensatory fitness effects from artefactual sources of selection have been underestimated and / or overlooked. Further, the last ML selection experiment represents the first study in this area that does not use the LH_M base population of *D. melanogaster* (reviewed in Thyagarajan *et al.*_a (chapter 2)). This heavy focus on a single strain of flies certainly restricts the generality of current findings regarding the species, and necessitates experimentation with more diverse stocks at the least, and more species ideally.

Sexual dimorphism

Lastly, there is the strategy of selection to disrupt intersex genetic correlations ($r_{g,m-f}$) for specific traits. The persistence of positive trait correlations in response to sexually antagonistic selection is an important component underpinning IaSC, preventing it's immediate resolution in the face of divergent selection (Bonduriansky & Chenoweth 2009, Stewart *et al.* 2010). It must be noted that (1) the evolution of dimorphism may not be a sufficient condition for the resolution of IaSC (Bedhomme & Chippindale 2007, Cox & Calsbeek 2009); and that (2) $r_{g,m-f}$ only accounts for additive intersex genetic correlations (and is sensitive to effective population size (N_e)), potentially ignoring non-additive pathways to

IaSC resolution (Kaufmann 2022). Even with this caveat, it is useful to study the constraints on evolved changes in sexual dimorphism (SD) in response to selection.

These selection experiments have pursued two distinct approaches: first, where experimenters select for increased SD of a trait within siblings of families (family-level selection); and second, where individuals in a population are selected in opposite directions for the same trait (individual-level disruptive selection). We identify 3 studies that conduct family-level selection on SD – on wing vein length in fruit flies (*D. melanogaster*) (Bird & Schaffer 1972), flower size in the white campion (*Silene latifolia*) (Delph *et al.* 2011), and body size in the seed beetle (*C. maculatus*) (Kaufmann *et al.* 2021); and 6 studies that employ individual-level disruptive selection – on abdominal bristle number in fruit flies (*D. melanogaster*) (Harrison 1953, Frankham 1968_b) and body size in mice (*Mus musculus*) (Korkman 1957, Eisen & Hanrahan 1972), flour beetles (*Tribolium castaneum*) (Tigreros & Lewis 2011), and fruit flies (*D. melanogaster*) (Stewart & Rice 2018).

All studies employing family-level selection found evolved changes in SD for the selected trait. Delph *et al.* find dramatic changes - $r_{g,m-f}$ approaching unity declined to zero within 5 generations in 2 out of 3 replicates. While Bird & Schaffer do not report $r_{g,m-f}$ they observe moderate changes in SD within a relatively short span of 15 generations of selection, which persisted even after selection was relaxed. Interestingly, in the case of Kaufmann *et al.* (2021) selection on male body size alone (up or down selection) as well as selection for increased SD produced evolved changes in SD within 10 generations of selection. Here, they identified pronounced Y-linked effects and some X-linked effects explaining the evolution of SD, in spite of a strong positive autosomal $r_{g,m-f}$. This was then confirmed by introgressing selected Y chromosomes into an otherwise isogenic background to recreate this effect.

Together, these studies suggest that SD adaption should be easily achieved in response to selection. However, Stewart & Rice (2018) raise an important criticism of this selection

strategy. First, they note that sexually antagonistic selection in natural populations is likely to be experienced at the level of the individual rather than through family-level selection. In the former scenario, the genetic architecture is the constraint on adaptation, whereas in the latter, selection acts directly to favour genetic architectures enabling sexual dimorphism. Second, family-level selection selects individuals likely sharing maternal, environmental, and non-additive genetic effects; which is expected to constrain phenotypic variation and substantially accelerate adaptation. Nevertheless, these studies show that solutions exist – genetic architectures segregating in populations that could evolve under appropriate conditions. Specifically, the Y-linked route to altered SD identified in Kaufmann *et al.* suggests a general mechanistic route to IaSC resolution. Such a finding could also explain studies that find correlated responses in sexual dimorphism when selection is applied to only one sex / concordantly to both sexes (Frankham 1968_a, Wilkinson 1993, Reeve & Fairbairn 1996, Delph *et al.* 2004, Duxbury *et al.* 2017). Further, Kaufmann *et al.* (2023) demonstrate that this evolved dimorphism results in a reduction in genetic variance for fitness, providing rare empirical evidence for IaSC influencing the maintenance of genetic variance.

What then, can we learn from individual-level disruptive selection? We find a mixed bag, with some studies finding moderate evolved changes in SD (Harrison 1953, Frankham 1968_b, Korkman 1957, Eisen & Hanrahan 1972), while others found no evolved response in SD or $r_{g,m-f}$ (Tigreros & Lewis 2011, Stewart & Rice 2018). It is worth noting here that all but Stewart & Rice (2018) work with very small N_e (≤ 20 mating pairs, with some as small as 2 pairs/generation), exposing the selection experiment to inbreeding effects and genetic drift. While this is adequate and perhaps even ideal in work motivated by commercial breeding interests, it is not particularly informative in the context of natural populations and their evolutionary trajectories. Overall, we conservatively interpret this literature as pointing to

substantial challenges in overcoming IaSC load in moderate evolutionary timescales, while noting a requirement for further work along these lines conducted on more traits and species.

Trait selection

Early inroads in this category came from Delph *et al.* (2004), who provided an indirect demonstration of SA effects through selection on flower size in *S. latifolia*. They found a strongly positive $r_{g,m-f}$ for flower size and a trade-off between flower size and number in both sexes. While they did not assay reproductive fitness of selected individuals, they identify a constraint on adaptation to the expected fitness optima in each sex. Ovule production is constrained by flower size, making larger flowers beneficial to female fitness. By comparison, males benefit from displays of numerous small flowers to maximize pollen transfer.

Berg & Maklakov (2012) showed that selection on male lifespan in the seed beetle *C. maculatus* produced adaptive responses within 5 generations, along with correlated responses in female lifespan, indicating a positive $r_{g,m-f}$. Here, lines selected for increased and reduced lifespan were explicitly tested for reproductive fitness. When tested, they found a negative sex by selection interaction, with long-lived lines improving female fitness at the cost of male fitness, and short-lived lines improving male fitness at the cost of female fitness. It is worth noting that this work is conducted with small N_e (4 mating pairs per line, per generation). In a follow up Berger *et al.* (2014) showed that this selection experiment affected an entire suite of functionally integrated traits. Short-life selection ‘masculinized’ metabolic rate, locomotor activity and body mass (besides lifespan), and long-life selection ‘feminized’ the same.

Mills *et al.* (2012) use selection on male testosterone (T) titres in bank voles (*Myodes glareolus*) to demonstrate a similar negative sex by selection interaction. Within a generation of breeding selected animals, high T lines showed improved male fitness at the cost of female fitness, while low T lines showed reduced male fitness and improved female fitness.

In an ingenious experiment, Plesnar-Bielak *et al.* (2014) conduct selection on a male-limited secondary sexual trait in the bulb mite *R. robini*. Males of this species develop into one of two reproductive morphs - fighters and scramblers. Fighter males carry a weapon - a thick and sharp third pair of legs used in male-male competition to mortally stab other males that distinguishes them from the scambler morph. While reproductive morphs are partially determined by juvenile condition (Smallegange 2011), they are known to be heritable (Radwan 2003). Having found no frequency dependent selection responsible for the maintenance of the two morphs (Radwan & Klimas 2001, Deere & Smallegange 2014), Plesnar-Bielak *et al.* predicted a role for IaSC in the maintenance of this trait polymorphism. Lines were selected to have exclusively fighter (F lines) or scambler (S lines) males, and were found to achieve a stable equilibrium after ~ 40 generations of selection, with the selected morph approaching frequencies of 90% in each line. When measuring correlated responses in females, they found that females from S lines lived longer and were more fecund regardless of which morph of male they were mated with – demonstrating that variants that produced fighter males resulted in low fitness females in spite of the male-limitation of the morphs and associated armament. Skwierzyńska *et al.* (2018) add nuance to these findings, by testing for correlated responses to male-morph selection in females under environmental stress. At higher temperatures, they find the same results as Plesnar-Bielak *et al.*, but at lower temperatures, this pattern reverses.

Overall, besides explicitly demonstrating IaSC associated with the traits studied, these studies point out the importance of correlated responses to selection and the constraints mediated through IaSC. Additionally, Plesnar-Bielak *et al.* demonstrate a mechanistic route to the maintenance of heritable variation through IaSC in the context of alternative reproductive tactics (ARTs).

IeSC EVOLUTION EXPERIMENTS

Rice (1996)'s demonstration of male adaptation to the arrest of female co-evolution marks the first evolution experiment to address IeSC and mate harm. Ever since, evolution experiments have typically studied IeSC by modulating opportunities for polyandry, with the help of monogamy or biased sex ratio treatments. These ideas have been reviewed previously (Hosken *et al.* 2009, Edward *et al.* 2010), and we briefly update this synthesis here, including literature published since.

Monogamy, sex ratio manipulations

The first such evidence of IeSC effects comes from Holland & Rice (1999), where reproductive outcomes for males and females were artificially equalized by enforcing monogamy selection in *D. melanogaster*. Monogamy lines saw a decline in male courtship, male induced mate-harm, and female survivorship post-mating, but an increase in net reproductive rate measured at the population level – demonstrating that the absence of IeSC helped overcome a fitness load. Similar methodologies (monogamy or sex ratio manipulation) have been applied in a variety of species (*Sepsis synipsea*, *R. robini*, *C. maculatus*, *D. melanogaster* and *pseudoobscura*...), to demonstrate direct evidence for IeSC in the form of male harm (Hosken *et al.* 2001, Martin *et al.* 2004, Crudgington *et al.* 2010, Nandy *et al.* 2013_b, Demont *et al.* 2014, Debelle *et al.* 2016) and female resistance (Wigby & Chapman 2004, Tilszer *et al.* 2006, Michalczyk *et al.* 2011, Nandy *et al.* 2014, Rostant *et al.* 2020, Kyogoku *et al.* 2023). These have also been used to study associated adaptations in the form of (1) post-copulatory processes like sperm competition (Pitnick *et al.* 2001, Linklater *et al.* 2007, Simmons & García-González 2008, Crudgington *et al.* 2009, Nandy *et al.* 2013_a) and manipulation of female remating (Crudgington *et al.* 2005, Hollis *et al.* 2019); and (2) correlated characters like body size (Promislow *et al.* 1998, Stångberg *et al.* 2020), genital

morphology (Cayetano *et al.* 2011), metabolic rate and locomotory activity (Garlovsky *et al.* 2022). Given that these treatments simultaneously modulate sexual selection and IeSC, not all characters described here are exclusively explained by conflict. While not uniform across all studies, polyandry and male-biased sex ratios generally tend to promote greater levels of mate-harm and mate-harm resistance. These tend to be associated with increased body size, locomotion and sperm competitive capabilities, which led to a fascinating chicken-egg debate in the early 2000s (Snook 2001, Wigby & Chapman 2004) that was finally settled through a re-analysis of original findings (Rice & Holland 2005).

While generally congruent, the lack of precise consistency in results across these experiments suggests that variation in the evolutionary histories and ecology of the species (and populations) tested could play a role in the adaptive response. Additionally, it has recently been argued that the difference between monogamy and polygamy treatments is more than just a difference in presence or absence of sexual selection / IeSC (Mishra *et al.* 2024). For instance, polyandrous selection can weaken natural selection on females through disproportionate harm targeted at high fitness females (Long *et al.* 2009). Such selection differences could also play a role in explaining these differences.

A variety of traits have been studied using these selection experiments, but justifiably in the context of sexual selection rather than IeSC, such as development time (Hollis *et al.* 2017), courtship traits (Snook *et al.* 2005, Hunt *et al.* 2012, Debelle *et al.* 2014, Chechi *et al.* 2022) and condition dependence of body size (Bath *et al.* 2023). While unlikely, it is possible that these traits too can be affected by IeSC, through pleiotropy or energetic trade-offs. Occasionally, traits may be routes to both energetic trade-offs and direct involvement in sexual conflict. Immune response is a good example of such a trait. Studies have demonstrated reduced investment (or indifference) in immune function in response to increased intensity of sexual selection / IeSC (McKean & Nunney 2008, McNamara *et al.*

2013, van Lieshout *et al.* 2014, Hangartner *et al.* 2015, Nystrand *et al.* 2018, Syed *et al.* 2020). In some species though, male traits like spiked genitalia or traumatic insemination can evolve through arm's race coevolution. In such situations, female wound healing and immune response to infections in the face of these injuries can undergo selection in arm's race coevolution (Bagchi *et al.* 2021).

A recent addition to this literature is the use of “evolve and re-sequence” experimental approaches in these selection experiments. In a pathbreaking first, Hollis *et al.* (2014) demonstrated that the elimination of sexual selection on males through monogamy feminizes expression in both sexes of *D. melanogaster*, with up and down expression changes in female- and male-biased loci respectively. Using a similar system of monogamy selected and elevated polyandry lines in *D. pseudoobscura*, Immonen *et al.* (2014) initially demonstrated that the treatment level differences had occurred in the transcriptome of virgin females, that included surprise increases in expression of some genes associated with male-specific functions. Veltsos *et al.* (2017) followed this with an analysis of the transcriptome in both sexes, and found that some genes follow the pattern described by Hollis *et al.*, but the majority display the opposite – a masculinization of gene expression in response to monogamy selection. These differences echo the outcomes of trait assays before them, where disparate lines of adaptation were seen in response to monogamy / sex ratio selection. Veltsos *et al.* (2022) then characterized the transcriptome pre- and post-mating, and found substantial differentiation between selection treatments in the reproductive tissues of virgin males and females, as well as a post-mating in the reproductive tract of females. Using the same selection experiment, Wiberg *et al.* (2021) report sequence divergence in many “islands” across the genome including regions related to sexually selected characters like mating behaviours, and an enrichment of sequence level divergence on the X chromosome. Most recently, Mishra *et al.* (2024) use a similar experiment in *D. melanogaster*, comparing

monogamy selected and control lines, and demonstrate that loci with pre-existing sex-biased gene expression (SBGE) are more likely to display expression changes in response to treatment differences. By contrast, alternative splice variant evolution was not enriched in SBGE loci. Further, they predicted a reduction of dimorphism under monogamy, but did not find evidence for the same. Lastly, they return to the findings of feminization from Hollis *et al.* (2014). They find (1) that while female body transcriptomes do evolve to be more feminized under monogamy, both male-biased and female-biased genes show increased expression in male body transcriptomes; and (2) no consistent pattern in either sex when considering head transcriptomes.

Lastly, theory predicts that cycles of IeSC co-evolution combined with allopatric separation can result in accelerated evolution of reproductive isolation (Rice 1998_b, reviewed in Gavrillets 2014). Briefly, when IeSC causes an arm's race between males and females, this rapid local co-adaptation occurs at loci influencing traits relevant to hybridization. Selection experiments have addressed this expectation by comparing levels of reproductive isolation (RI) evolving between replicate populations at various levels of IeSC. Again, while some do not (Wigby & Chapman 2006, Bacigalupe *et al.* 2007, Gay *et al.* 2009, Plesnar-Bielak *et al.* 2013), some experiments do find evidence for IeSC accelerating prezygotic RI (Martin & Hosken 2003, Hosken *et al.* 2009, Syed *et al.* 2017) but not postzygotic RI (unpubl. Chatterjee 2017, Thyagarajan 2018) as predicted.

INTERACTION EVOLUTION EXPERIMENT

IaSC and IeSC have classically been delineated as independent processes of conflict (cf. Schenkel *et al.* 2018). This framework may ignore ongoing interactions between the two, emerging when traits engaged in arms race coevolution under IeSC are not sex-limited. For instance, this may induce additional fitness loads directly or pleiotropically on the opposite sex through IaSC. Such costs may make novel armaments prohibitive, de-escalating the arms race to an equilibrium state. For instance, Plesnar-Bielak *et al.* (2014) demonstrate that the male-limited armament of fighter bulb mites carry an IaSC load. While this is a weapon used for male-male combat rather than mating interactions, it is easy to imagine a direct parallel where such a structure evolves in an arm's race over mating rate conflict. Alternatively, depending on the genetic architecture of traits involved, increased strength of IeSC selection could act to ameliorate the IaSC fitness load. Pennell *et al.* (2015) model a scenario where reproductive traits are involved in both types of conflict, and find support for the verbal model that IaSC is capable of restraining IeSC in cases with between-sex pleiotropic trait expression, under conditions where mating traits are near evolutionary equilibrium. However, when mating traits are further displaced from equilibrium, such interactions can also prolong arms race dynamics with alternative phases of IaSC build-up and resolution.

To our knowledge, there are no selection experiments that explicitly evaluate potential interactions between the two modes of sexual conflict. We include a quantitative genetic approach here as an exception to the scope of this review here. Geeta Arun *et al.* (2023) assay reproductive fitness of males and females in *D. melanogaster* hemiclone lines at different intensities of IeSC (modulated using sex ratio). Line-wise fitness estimates obtained are used to estimate $r_{g,m-f}$ for fitness (cf. Connallon & Matthews 2019) and a measure of proportion of sexually antagonistic fitness variation. They find that the fitness displays a positive $r_{g,m-f}$ at all intensities of IeSC, but that low intensity IeSC produced a nominally lower $r_{g,m-f}$ and greater

proportion of sexually antagonistic variation. This data suggests a possibility of amelioration of IaSC with greater intensity of IeSC. Geeta Arun *et al.* identify two non-exclusive routes to such IaSC amelioration – (1) if changes in IeSC resulted in changes in male and female fitness functions mediating IaSC, and (2) if traits in each sex mediating IeSC shared a positive $r_{g,m-f}$. It is worth noting that the fitness assay design for male reproductive fitness might be noisy, due to artefacts in the hemiclinal analysis system described earlier. A fascinating follow-up could address levels of IaSC observed in lines selected over time at various intensities of IeSC, modulated by monogamy / sex ratio.

Here, we also speculate about a second design to study IaSC-IeSC interactions. Theory predicts that IaSC intensity is reduced by novel environments (Connallon & Clark 2014, Connallon & Hall 2018), creating poorly equilibrated / “off-peak” populations (*sensu* Fricke & Arnqvist 2007, Long *et al.* 2012). For instance, Long *et al.* find that high fitness males (identified in the context of sexual selection) produce high fitness daughters in off-peak populations, but low fitness daughters in on-peak populations, demonstrating changes in IaSC as a consequence of disturbance from equilibrium. On & off peak populations (at varied levels of IaSC) could be used to derive selection lines with varied intensities of IeSC. With such lines, it would be possible to study the IaSC-IeSC interaction effects. However, we note that it is necessary to test off-peak populations rigorously for changes in IaSC prior to IeSC selection (cf. Martinossi-Allibert *et al.* 2018). Such selection experiments have been conducted before to study the role of sexual selection in accelerating adaptation to a novel environment (reviewed in Rowe & Rundle 2021). However, these studies do not assess IeSC and IaSC loads, preventing us from retrospectively commenting on such interactions. A (perhaps fatal) caveat worth noting is that such a design could create a confound between IaSC intensity and population equilibration.

CONFLICT IN ITS ECOLOGICAL SETTINGS

The variability of results and the narrow parameter space of classical IeSC selection experiments suggests a need for a greater understanding of IeSC in its natural settings (Svensson 2019). Arbuthnott *et al.* (2014) initially identified this need to study local adaptation to ecological settings and their impact on IeSC. Using arbitrary sources of selection (exposure to chemicals), they demonstrated that locally adapted populations of *D. melanogaster* follow independent IeSC trajectories (measured using levels of male mate harm and female resistance), but trajectories that could be predicted in part by their respective ecologies. A growing list of studies have since focussed on evolved changes in / interactions with IeSC in response to various selection pressures. Gomez-Llano *et al.* (2018) show that changes in species composition can ameliorate male harm at low conspecific densities in the banded demoiselle (*Calopteryx splendens*). Łukasiewicz (2020) investigated changes in juvenile diet in the ARTful mite *Sancassania berlesei* but find no differences in intensity of IeSC. Canal *et al.* (2022) find that metapopulation structure interacts with IeSC intensity in explanations of evolved behavioural changes in *C. maculatus*. Abiotic variation in factors like temperature (García-Roa *et al.* 2019) and the complexity of physical environments of selection (that modulate opportunity for female control over mating) (Yun *et al.* 2017, 2021) have been shown to ameliorate male harm in *D. melanogaster*. Natural selection acting on traits elsewhere can result in correlated changes in IeSC – for instance, Robinson *et al.* (2023) (also see Mital *et al.* 2021) find correlated changes in female resistance in response to life history selection in *D. melanogaster*, resulting in asymmetrical prezygotic RI between populations selected in opposite directions.

Barring amelioration in off-peak populations, little is known about the influences of ecology on IaSC. Skwierzyńska *et al.* (2018)'s work (discussed above) is the single exception to this trend. An increase in *D. melanogaster* body size in response to masculinizing selection

in Thyagarajan *et al.* (in prep) rather than the expected reduction (Prasad *et al.* 2007), could be interpreted as a consequence of ecology (cage based maintenance as against vial based maintenance), but alternate explanations are also possible there.

In the absence of empirical findings, theoretical advances shed light on potential ecological influences on IaSC. Flintham *et al.* (2021) model IaSC under various dispersal scenarios, and predict that kin competition in the philopatric sex biases intralocus conflict in favour of the more dispersive sex. Additionally, they find that inbreeding should reduce IaSC disproportionately on the X chromosome relative to autosomes. Lerch *et al.* (2020) use a spatially explicit individual based model to study mate choice and territory establishment, and find that territory edges promote IaSC – suggesting sexually antagonistic consequences for populations experiencing habitat fragmentation.

These studies provide valuable insights into variations in the sexual conflict canon, and demonstrate the need for a dialectic between ecology and evolutionary theory to arrive at a complete picture.

SEXUAL CONFLICT IN HERMAPHRODITES

While the focus of sexual conflict (and IaSC) studies is typically on dioecious species, sexual conflict can also play out even when individuals have more than one sex function (Prasad & Bedhomme 2006, Schärer *et al.* 2014). Sexual conflict in hermaphrodites can be thought of in terms of analogues of the canonical forms of sexual conflict. Here, IeSC involves conflict over mating role (male-like or female-like), analogous to mating rate conflict and mate harm. For instance, individuals can evolve to alter their own, or manipulate the mating role of their partner to optimize their own resource allocation in reproduction (reviewed in Bedhomme *et al.* 2009). Like gonochorists, post-copulatory control over the sperm that fertilizes the sperm recipient can be an important IeSC battleground (reviewed in Beekman *et al.* 2016). Individuals can also undergo antagonistic pleiotropy between their sex functions, which is somewhat analogous to IaSC (reviewed in Abbott 2011). However, two important differences follow – (1) SA effects function like energetic trade-offs within individuals, in the form of resource allocation to different sex functions and (2) unlike dioecious species, the evolution of sex-limitation of traits may not be a viable resolution of IaSC, especially for simultaneous hermaphrodites.

Selection experiments on hermaphrodites have utilized some strategies used in dioecious species, and some novel hermaphrodite specific approaches. We identify studies conducting monogamy (mate-limitation) selection to modulate the intensity of sexual selection / IeSC, sex-limited middle-class-neighbourhoods (MCN) to select on only one sex function, forced-selfing, forced-outcrossing, and “pick-a-lane” selection that restricts fitness to one sex function while ignoring the other. Predominantly, these studies are interested in evolved changes in resource allocation to sex function. Before describing these experiments though, it is relevant to acknowledge a body of work on plastic responses to ‘local mate competition’ (LMC) (Hamilton 1967), that describes increased investment in male (sperm

donor) function at the cost of female function in larger groups (Raimondi & Martin 1991, Trouvé *et al.* 1999, Schärer & Wedekind 2001, Lorenzi *et al.* 2005) even prior to selection.

Monogamy selection

Janicke *et al.* (2016_b) subjected the hermaphroditic flatworm *Macrostomum lignano* to monogamy for 20 generations. Here, they predicted the evolution of greater investment in female function in selected animals relative to controls maintained in octets. Individuals evolving under monogamy were found to have no differences in sex allocation (measured as testes and ovary size), but did display reduced investment in sperm bristle length and morphology. The majority of traits assayed displayed significant plastic phenotypic responses to change in group size, rather than significant responses to selection.

Obligate selfing

Working with the freshwater snail *Physa acuta* Noël *et al.* (2016) take mate limitation a step further than monogamy by selecting individuals to exclusively reproduce through selfing, completely eliminating selection on male-function. Much like Janicke *et al.*, Noël *et al.* find no change in sex allocation in selected lines relative to controls after 20 generations of selection. However, resistance to selfing in the form of reduced latency to selfing in selected lines. Interestingly, they report on substantial recessive deleterious variation being purged through exposure to selection in the obligate selfing treatment. This was measured in the form of inbreeding depression (ID) experienced in selected and control lines, with control lines experiencing greater ID. Like Martinossi-Allibert *et al.* (2019), this study was conducted on a synthetic population created by pooling variation from iso-individual lines. It is possible that the newly formed population is not equilibrated, resulting in the selection outcomes observed.

Middle class neighbourhoods

Using the same *P. acuta* base population, Bonel *et al.* (2018) create both monogamy selection lines (labelled F lines) and female MCNs (labelled M lines). F lines are expected to undergo increased allocation to female function, at the cost of male function under mate limited conditions. Fitness achieved through male function was allowed to vary and undergo selection in M lines, potentially at the cost the female function, where fitness was constrained to equity. In the control (C) lines, both male and female fitness function could undergo selection. Evolved changes were assayed thrice, in generations 20, 35 and 37 with some variation in results. Summarized briefly - the majority of traits assayed displayed no selected differences between F, M and C lines. However, they observe a decrease in juvenile survival (a sexually concordant trait) and male reproductive performance in the F lines. M lines did not differ significantly from controls. Overall, this is interpreted as a result of selection eliminating sexually concordant deleterious variance. While these variants continue to segregate in the absence of sexual selection (under monogamy), sexual selection on male function alone is sufficient to select against them, rendering them indistinguishable from the control. This pattern would be consistent with insufficient evolutionary equilibration prior to selection, as discussed above.

Obligate outcrossing

Palopoli *et al.* (2015) use *fog2* mutants in the roundworm *Caenorhabditis elegans* to force hermaphrodites to behave as females by blocking their capacity for sperm production. They then study the evolved consequences of breeding system change for 60 generations in these high-competition (HC) lines relative to *fog2*- control lines with no males maintained in them. HC males adapted within 30 generations to have larger sperm and perform better as sperm competitors. Additionally, they were more harmful than controls when mated to tester

hermaphrodites, measured in the form of post-mating mortality. Here, they do not characterise sex allocation in the form of testes and ovary sizes. Working independently on a *fog2* system, Antol *et al.* (2022, 2023) do not replicate these findings, with no differences identified between selected and control populations. However, they also note that this selection experiment overlaps with laboratory domestication effects in both the outcrossing and control lines, which perhaps obscures the effects of interest.

Pick-a-lane selection

Norden *et al.* (2023) bring us back to *M. lignano* with an ingenious yet simple selection experiment. Here they use a phenotypic marker to identify offspring produced by the sex function of interest of target individuals. With the capacity to identify these offspring, they demand that their selected animals “pick a lane” – selecting only on male-function (M lines) or female function (F lines) while culling the remaining offspring. In keeping with experiments described above, Norden *et al.* find no differences in sex allocation measured in the form of testis or ovary morphology. However, they find a rapid increase in fitness via the female function, at the cost of fitness via the male function in F lines relative to controls.

Overall, we found that selection on sex function did not result in changes in sex allocation, perhaps due to a narrow definition of sex allocation in the form of testis and ovary size. While these traits have received attention because they do respond to changes in local mate competition, the degree of trait plasticity seems to preclude adaptive responses in response to selection. That said, selection on sex function does result in adaptive changes in the predicted direction, measured in the form of reproductive fitness and gamete level investment. Likewise, preliminary breeding system changes in the form of reduced resistance to selfing can evolve as a response to selection.

SEXUAL CONFLICT IN HAPLO-DIPLOID SYSTEMS

A second taxonomically abundant yet insufficiently studied group are haplo-diploid species. Haplo-diploid systems come in 2 broad flavours – (1) arrhenotokous reproduction where fertilized embryos result in diploid females, and unfertilized embryos produce haploid males; and (2) paternal genome elimination where somatically diploid males eliminate their paternal DNA in their germline. In the absence of substantial empirical research, our understanding of these systems has historically depended on parallels to the haplo-diploid sex chromosome systems (Hedrick & Parker 1997). Besides the obvious consideration that sex chromosomes represent a portion rather than the whole genome, we also need to consider that these species are a rich playground of mating, social, and caste system evolution. Amongst other ‘deviations’ (from the diplo-diploid standard), (1) monandrous mating systems with female biased broods are extremely common, (2) male genomes are exclusively inherited from dams and passed on to daughters without recombination, and (3) eusocial populations with highly related individuals are common (reviewed in Kraaijeveld 2009, de la Filia *et al.* 2015).

Taking some of these considerations (time spent by alleles in females vs males, exclusive father-daughter inheritance) into account, Klein *et al.* (2021) first extend sex chromosome based modelling of invasion and fixation criteria for sexually antagonistic (SA) alleles to haplo-diploid systems. Echoing findings from Rice (1984), they find that haplo-diploid systems facilitate easy invasion for dominant-female-benefit and recessive-male-benefit SA alleles, but constrain them from fixation – promoting stable SA polymorphisms. Hitchcock *et al.* (2022) push this synthesis forward leaps and bounds, explicitly considering ecology of these species in the form of increased levels of sib-mating, viscous population structures with localized resource competition, and sex-specific dispersal systems. They find that increased sib-mating in general promotes feminization, or the ease of invasion of female-

benefit variation, with the strongest effect in paternal genome elimination systems. Greater local resource competition on the other hand enables masculinization. Dispersal produced qualitatively similar results to changes in sib-mating intensity. Given male dispersal, species that allowed mating prior to female dispersal enabled greater levels of feminization than those that mated post female dispersal.

By contrast, IeSC in haplo-diploids has received less attention – perhaps due to the preponderance of monandrous systems with low-intensity conflict. However, like hermaphroditic systems, haplo-diploid species are a focal point of interest in responses to local mate competition. Female interests in brood sex ratios are expected to vary with local mate competition (LMC). When fewer foundresses contribute to a population patch (high LMC), a female-biased brood sex ratio maximizes number of grand-offspring (scaling with number of daughters) and maximizes fitness for sons through low intensity of sexual selection. However, when the number of foundresses increase (reducing LMC), females are incentivized to produce more sons that can “capture” a greater proportion of the grand-offspring. Since paternal fitness always accrues exclusively through daughters; parental interests diverge, and control over brood sex ratios can be a flash-point of IeSC (reviewed in de la Filia *et al.* 2015). In a more elaborate treatment, Hitchcock *et al.* (2022) model conflict around brood sex ratio while explicitly accounting for levels of sib-mating, which can increase paternal interest in sons.

To our knowledge, there are no selection experiments that test IaSC predictions in haplo-diploid systems. With such a rich set of explicit predictions, we believe that this area represents valuable low hanging fruit for selection experiments.

We identify one selection experiment focussed on adaptive responses to intensity of LMC. Using the spider mite (*Tetranychus urticae*) Macke *et al.* (2011) first demonstrate that brood sex ratios do respond to intensity of LMC selection (modulated through number of

foundresses). While LMC can induce changes in brood sex ratios through phenotypic plasticity, only low LMC lines display a capacity to produce both female-biased and even broods, as against high LMC lines, that produced female-biased broods regardless of LMC conditions they were tested in. Macke *et al.* (2014) follow up to show that a greater intensity of LMC selection results in an improved ability to manipulate the brood sex ratio when mated, in both males and females. Importantly, this is one of the first lines of evidence of male capacity to manipulate sex allocation.

CONCLUSION

Sexual conflict selection experiments have made rapid strides in recent years, demonstrating predictable trait responses to modulation of Ia- and IeSC. While the majority of findings are in concert with expectations, we continue to find some surprises, and different responses to very similar selection experiments. In some cases, this calls for an improved understanding of the interactions between our selected populations and the selection treatments. In others, we advocate for greater caution in experiment design, in the form of accounting for artefactual sources of selection and allowing for evolutionary equilibration in populations studied.

Further, low-hanging fruit in the form of selection experiments remain available for the picking. Specifically, we reiterate the need for studies of conflict (1) in its ecological setting and (2) in haplo-diploid species. A little further from reach on the same tree hang ripe fruit – studies on (1) the maintenance of genetic variance through sexually antagonistic balancing selection and (2) the outcome of interactions between Ie- & IaSC.

CHAPTER 2

Mixed evidence for intralocus sexual conflict explored through male-limited selection in

Drosophila melanogaster

ABSTRACT

Due to the shared ubiquity of sex-specific selection pressures and positive genetic trait correlations between the sexes, intralocus sexual conflict (IaSC) is predicted to be common and relevant to the evolutionary genetics of sexually reproducing populations. Direct tests of the contributions of IaSC to these phenomena are challenging to conduct, and limited. In this study, we utilize a sex-limited selection experiment, designed to subject haplotypes of *Drosophila melanogaster* to selection for male fitness without opposing selection acting on female fitness. In contrast with previous studies employing this technique, we find that male fitness of haplotypes subject to male-limited (ML) selection are not significantly better than their matched controls (MC). Males from ML lines do not outperform controls in mate choice trials, fecundity induction or sperm offense. They show no signatures of driving sex chromosomes (measured in the form of biased sex ratios). Genetic variation for male fitness was reduced by selection, but this was only surveyed in one replicate population pair and included a potential artefact in the protocol. On the other hand, female fitness in ML lines declined relative to controls as predicted by sexually antagonistic variation. These results bring into question the ubiquity of IaSC in *D. melanogaster* populations. However, we also discuss some likely artefacts that may obscure our ability to detect IaSC altogether.

INTRODUCTION

Anisogamy of the sexes triggers an adaptive cascade that causes sex-specific selection pressures, resulting in different morphological, life-history, reproductive and ecological strategies. Despite sex specific selection pressures being common, most traits genes show positive genetic correlations between the sexes (Poissant *et al.* 2010). These correlations are expected to act as constraints the evolution of dimorphic responses to selection. This inability to evolve to the fitness optimum in either sex results in a conflict between the sexes, labelled intralocus sexual conflict (IaSC).

The empirical examination of IaSC is a field in relative infancy. After the formalisation of theory in the 1970s (Kidwell *et al.* 1977; Parker 1979, Lande 1980), early experimental evidence for the existence of IaSC was provided by Chippindale *et al.* (2001) in the form of a negative genetic correlation for reproductive fitness between the two sexes. This measure of intersex (or cross-sex) genetic correlation for fitness ($r_{w,g,m-f}$) has since been used to investigate the existence of IaSC, with negative measures of $r_{w,g,m-f}$ treated as evidence for IaSC (cf. Connallon & Matthews 2019). These calculations are typically applied to forms of cytogenetic cloning (hemiclinal analysis), half-sib breeding designs, or sex-specific parent-offspring fitness data, and have been used to identify a number of species undergoing IaSC including fruit flies, seed beetles, zebra finches, ground crickets, meal moths, plants, lizards, snakes and red deer (reviewed in Bonduriansky & Chenoweth 2009; Poissant *et al.* 2010; Connallon & Matthews 2019).

Besides measurements of intersex genetic correlations for fitness at the species / population level, a second key focus of this literature has been on specific traits that are potential fulcrums of sexual antagonism. In the absence of sex-specificity of traits, the evolution of adaptations to sex-specific selection pressures is constrained by the shared

genome (Lande 1980). While sex-specific adaptation may be accomplished through a number of genomic mechanisms, such as duplications, sex chromosomes, imprinting, or in cis-regulation, even dimorphic traits may harbour unresolved sexual conflict. Through measurements of extant trait values and fitness optima (estimated using selection gradients), dimorphic traits such as body size, wing shape, locomotory activity and development time (amongst others) have been shown to have SA fitness effects (reviewed in Bonduriansky & Chenoweth 2009; Cox & Calsbeek 2009; Singh & Punzalan 2018).

While a growing list of studies catalogue the existence of IaSC and the constraints placed upon trait dimorphism in different species, empirical studies that test theoretical predictions of the consequences of IaSC with respect to the maintenance of genetic variance and the genomic locations of sexually antagonistic loci are limited.

The interest in the distribution of SA loci stems from contradictory theoretical predictions about the SA loci enrichment on the haplodiploid sex chromosome based on differing assumptions about allele dominances (Fry 2010). Assuming identical allelic dominance parameters in males and females, Rice (1984) predicts an enrichment of SA variation on the haplo-diploid X chromosome. In a more general model that considers all possible dominance combinations, Kidwell *et al.* (1977) shows that a less restrictive parameter space allows for similar invasion conditions on autosomes and X chromosomes. Empirical research has so far suggested that the X chromosome does indeed harbour more SA loci than randomly expected (Gibson *et al.* 2002; Pischedda & Chippindale 2006; Long *et al.* 2012; Ruzicka *et al.* 2019, Wong & Holman 2023, but see Lund-Hansen *et al.* 2020, Abbott *et al.* 2020). However, Ruzicka & Connallon (2020) suggest that quantitative genetic methods attempts at identifying SA loci are likely to overestimate the enrichment of SA loci on the X chromosome, due to asymmetric inheritance pattern of X chromosomes. Further, recently unearthed evidence for sex-specific dominance reversals (Grieshop & Arnqvist

2018, Pearse *et al.* 2019, Geeta Arun *et al.* 2021, reviewed in Grieshop *et al.* 2024)

contributes weight to Kidwell *et al.*'s assumptions, and predictions of random distribution of SA loci across autosomes and sex chromosomes.

There is little empirical work detailing the maintenance of genetic variance through SA polymorphisms. As a form of balancing selection acting upon the genome constitutively, IaSC is expected to maintain polymorphisms and contribute to genetic variance for traits related to fitness. However, while the conditions for sexually antagonistic selection are nearly ubiquitous, the conditions for their maintenance may or may not be met in nature. Rapid environmental heterogeneity can cause maladaptation, which can align the selection pressures acting on the two sexes, but gradual environmental fluctuations are likely to stabilise antagonistic variation (Connallon & Hall 2016). Furthermore, the “efficacy” of antagonistic selection (i.e., its tendency to dominate over genetic drift) is extremely weak relative to classical models, such as directional selection and over-dominance (Connallon & Clark 2012). Ruzicka *et al.* (2019) show that the SA loci they identify in a lab population display signatures of balancing selection (measured using Tajima's D and minor allele frequency measurements) and identify similar signatures in distantly related populations of fruit flies, suggesting stable balancing selection over long periods of evolutionary time at these loci. More recently, Kaufmann *et al.* (2023) show that artificial sexually antagonistic selection on body size maintains greater genetic variance than sex-limited selection.

At the sequence level, the last few years have produced some remarkable indexing of sexual antagonism on the genome. Some studies have used the *D. melanogaster* hemiclinal analysis system, coupled with statistical techniques to extract an ‘antagonistic fitness axis’ along which they track expression differences (Innocenti & Morrow 2010, although see Rowe *et al.* 2018), and sequence variants (Ruzicka *et al.* 2019; also see Hill *et al.* 2017). Wong & Holman (2023) approach the same problem using fitness measures and gene

expression data for both sexes from the *Drosophila* Genetic Reference Panel. In other species, the absence of such panels and cytogenetic cloning techniques has produced alternative approaches to identifying SA loci. Recently, Dagilis *et al.* (2022) describe a technique to date recombination suppression between sex chromosomes and look for the accumulation of SA polymorphisms in the newly formed pseudo-autosomal region.

A common tactic has been to look at modes of resolution of IaSC to work backwards onto loci of antagonism. Sex linkage and sex-biased gene expression ranging from mildly biased expression to sex-limited expression can provide a direct form of intra-locus sexual conflict resolution, by creating the genetic architecture for phenotypic dimorphism (Day & Bonduriansky 2004; Otto *et al.* 2011; Jordan & Charlesworth 2012; Kirkpatrick & Guerrero 2014; Mank 2017). As a consequence, loci that display such patterns of altered expression are of especial interest as potential sites of ongoing sexual conflict, and have been shown to exhibit signatures of balancing selection (Qiu *et al.* 2013; Cheng & Kirkpatrick 2016; Guirao-Rico *et al.* 2017; Wright *et al.* 2018; Sayadi *et al.* 2019). Wright *et al.* 2018 and Sayadi *et al.* 2019 utilise indicators of balancing selection in conjunction with sex-specific expression data to predict loci under IaSC in guppies and find especially interesting patterns. They show that male-biased (in expression) genes and female biased genes undergo different levels of balancing selection on average, with female biased genes undergoing much higher levels of balancing selection, suggesting that expression changes in loci that are found to be male-biased are generally more successful at resolving IaSC.

In this study, we look to a sex-limited selection protocol (developed by Rice (1996) and pioneered as a tool to study IaSC by Prasad *et al.* (2007)) to address these questions. Using the lack of molecular recombination in *D. melanogaster* males and an independent population of “clone-generator” (CG) females carrying compound-X chromosomes (XXY karyotype) and autosomal translocations, we are able to reliably passage haplotypes containing an X

chromosome and all autosomes barring *D. melanogaster*'s dot (4th) chromosome hemiclonally from father to son. Removed from the influence of selection acting upon the female function, we predict that this population of haplotypes will experience a knock-down of IaSC, increasing the frequency of male benefit alleles at SA loci at the cost female benefit alleles. This should result in (1) a phenotype of increased male fitness at the cost of female fitness, (2) masculinization of partially dimorphic traits in both males and females (as in Prasad *et al.* 2007, Abbott *et al.* 2013), (3) a reduction of genetic variance for fitness in males, (4) sequence level divergence between selected and control populations with changes in signatures of balancing selection and (5) changes in patterns of sex biased gene expression.

This male-limited (ML) system holds advantages over other contemporary systems used in the literature. While clonal and breeding designs allow us to study the standing genetic variance for fitness in a population, they only provide us with a meaningful study of intralocus sexual conflict through the usage of correlational analysis using many sampled lines. To probe the system past fitness and identify SA polymorphisms on the genome, there is a need for statistical manipulations, such as the extraction of an antagonism index (Innocenti & Morrow 2010; Hill *et al.* 2017; Ruzicka *et al.* 2019). Further, it is not amenable to examination of other phenotypical trait variances and corresponding analysis. In contrast, the ML selection regime (1) provides a direct experimental manipulation rather than a correlational analysis, (2) allows for statistical simplicity and strength at smaller sample sizes through the amalgamation of male-benefit alleles across many loci in the same individuals, increasing signal-noise ratio and (3) allows us to probe adaptive responses in the phenotype when released from positive trait intersex genetic correlations.

That said, the ML system also has caveats that need to be explicitly stated, stemming from the genetic constructs used in CG females, and the use of CG females themselves. Firstly, the use of females in “arrest” (Rice 1996) (females are sourced from a different

population and cannot co-adapt with the males they are exposed to) could result in adaptations specific to these females. Within the genome, the usage of the compound-X (XXY) females to reverse the parent of origin of sex chromosomes may carry selection costs (Abbott *et al.* 2013). (1) Cyto-nuclear interactions between CG female cytoplasm and male nuclear DNA – Given that the XXY females cannot be backcrossed to standardize their cytoplasm using the control population, there remains a possibility for interactions between the cytoplasm of the CG females and the nuclear DNA of the focal males, in the form of mito-nuclear interactions. (2) Maternally inherited Y chromosomes may have undergone feminization or mutation accumulation effects of being expressed in female organisms 50% of the time, which may in turn play a role in the selective environment in which the ML flies are reared. Likewise, the translocated autosomes inherited from these females could produce fitness effects that induce counter adaptations over the course of the selection experiment. Lastly, given that selection acts upon exclusively haploid genomes, there is scope for adaptation to take place through dominance effects, or dosage compensation-like effects. Nevertheless, previous studies have found the system to be a valuable resource despite these artefacts.

Besides the interest in critical open questions, this selection experiment provides a valuable novelty to IaSC studies conducted using *D. melanogaster*. An unfortunate consequence of a tight knit research community is the restriction of studies concerned with IaSC to a limited number of stock populations. Specifically, the LH_M's (LH-moderate density) adapted from Larry Harshman's LH stock dominate this field (Rice 1996, Rice 1998, Chippindale *et al.* 2001, Gibson *et al.* 2002, Byrne & Rice 2005, Lew & Rice 2005, Lew *et al.* 2005, Linder & Rice 2005, Pischedda & Chippindale 2006; Prasad *et al.* 2007, Long *et al.* 2007, Bedhomme *et al.* 2008, Rode & Morrow 2009, Innocenti & Morrow 2010, Abbott *et al.* 2013, Collet *et al.* 2017, Hill *et al.* 2017, Ruzicka *et al.* 2019, Abbott *et al.* 2020, Lund-

Hansen *et al.* 2020, Manat *et al.* 2021, Lund-Hansen *et al.* 2022). Interestingly, Collet *et al.* demonstrate that even recently separated populations (~ 200 generations) of LH_M display differences in sexual antagonism. While results from Ruzicka *et al.* (2019) suggests some generality in these findings, it is useful to affirm this notion by conducting experimental work on an alternative baseline populations. Here, we conduct a selection experiment on control-old (CO) (derived from the *Ives* (IV)) populations that have been used as a long-term evolution experiment to study life history traits, correlated adaptations, and speciation (Rose *et al.* 1992; Chippindale *et al.* 1997; Chippindale *et al.* 2004; Xiao *et al.* 2019; Robinson *et al.* 2023).

We report on the competitive reproductive fitness (CRF) of males and females from selected and control lines. Using an improved breeding design, we strip test individuals of all artefacts deriving from the selection experiment. To study contributions to CRF, we further examine test males for mating success, fecundity induction, sex ratio drive and sperm offense. Lastly, we use a hemiclonal analysis to study genetic variance for reproductive fitness in males and females from selected and control populations, and use this design to report on the intersex genetic correlation for fitness in selected and control populations.

As a proof of principle of ML selection, we predict improved male fitness and reduced female fitness for selected haplotypes. Previous work suggests that this improved male fitness should be a consequence of more attractive males that are consistently mated (even with reduced courtship efforts, Bedhomme *et al.* 2008). Due to the reduced genetic variance for the already male limited sperm traits (Friberg *et al.* 2005, Bjork *et al.* 2007, Jiang *et al.* 2011), we anticipate that the improvement of male fitness will not evolve through improved fecundity induction or sperm competitive ability (although see Rice 1996, Rice 1998). Previous work reports no precedent of driving sex chromosomes, and the likelihood of such a mutation is low, but the selection experiment should strongly select for a driving ML-X

chromosome by design should it arise. Lastly, we expect ML selection to erode balanced polymorphisms at SA loci through directional selection for male benefit variation, resulting in reduced genetic variance for male fitness.

METHODS

ML Selection

Source populations

Our experiments employed selected and control populations (COs) originating from the IVs. These in turn were initiated in 1975 from a sample of wild-caught flies in South Amherst (MA, USA), and maintained under standardized culture conditions for ~500 generations since 1980 (Rose 1984). Three replicate CO populations (CO₁, CO₃, CO₅) (traditionally kept separately on discrete 4-week generation cycles since 1989,) were arbitrarily chosen from the five replicate CO's and were maintained in a 2-week cycle (day 1-11 in vials, followed by days 12-14 in ~ 3 litre population cages with food in a petri-dish) for 24 generations. After 24 generations, a male-limited (ML) line and matched-control (MC) line was derived from each of the three replicate populations. These were correspondingly labelled ML₁-MC₁, ML₃-MC₃, and ML₅-MC₅. All populations were maintained under standard laboratory conditions: moderate density (larval density of 80 - 100 larvae / vial), 25°C, 12:12 light:dark cycle on banana/agar/killed-yeast medium, and with a census population size of ~1,000 haploid genomes per generation.

Selection treatment

ML selection lines were established nearly identically to the protocol described in Prasad *et al.* (2007) (fig. A1a). Briefly, 1,000 males from the replicate base population were mated to “clone-generator” (CG) females that carried a compound X(C(1)DX, *y, f*), a Y chromosome and a homozygous-viable translocation of the two major autosomes (T(2 : 3)*rdgc st in ri p^p*). Using the genetic markers for eye colour, the absence of molecular recombination in males, and the aneuploid mortality experienced by animals inheriting uneven combinations of translocated autosomes, we select sons carrying a haploid genome

from their sire, and repeatedly cross these sons to CG females to passage these haploid genomes under conditions of ML selection. 1000 such males were collected under light CO₂ anaesthesia each generation on day 10-11 post oviposition (PO) and were combined on day 12 with virgin CG females of matched age in 3 cages (333-334 males and females in each cage). Eggs were collected from these cages on day 14 at a density of 320-400 eggs per vial, to achieve a larval density of 80-100 per vial after accounting for aneuploid viability costs. To match these conditions, 500 males (1000 haploid genomes) from each MC line collected under light CO₂ anaesthesia each generation on day 10-11 PO and combined on day 12 with virgin MC females in 2 cages (250 pairs per cage). Eggs were collected from these lines at a density of 80-100 per vial, with 20 vials dedicated to females and virgin collection and 20 to male collections.

Recombination Box

In the absence of molecular recombination in males, and the artificial prevention of independent assortment of chromosomes, the ML selection regime is not only sex-limited, but also asexual in propagation, exposing it to reduced rates of adaptation and genetic hitchhiking. We follow the system described in Prasad *et al.* (2007) and express some randomly selected pairs of ML haploid genomes in female flies each generation to facilitate recombination and limit hitchhiking and background selection. These recombinant (RC2) females carry completely ML derived genomic content and are used to dam male offspring with recombined DNA that are re-introduced into the ML population the next generation.

To facilitate recombination in each generation, we collected 50 ML males from each replicate population and crossed them to virgin CG females carrying an additional dominant eye colour marker (bw^D). Sons derived from this first recombination cross (RC1) carrying haploid genomes of interest are brown eyed due to their single copy of bw^D . 50 sons (RC1

males) were crossed to control females, and daughters carrying two ML haploid genomes (identified through absence of bw^D) from this second recombination cross (RC2) are collected as virgins to establish a line of females that receive ML genetic content through backcrossing with RC1 males. Within 4 generations of backcrossing these females are ~95% ML in genome content. In each generation after the first generation of the experiment, 70-80 virgin RC2 females were collected and crossed to 50 RC1 males. 50 male offspring from this cross (RC2 males) carrying haploid genomes of interest (rather than translocated autosomes - identified through absence of bw^D) from RC1 fathers and recombined gametes from RC2 mothers are re-introduced into the selected population from which the RC was derived (fig. A1b).

Experimental animals

We expressed the target (ML and MC) haploid genomes in male and female flies, with an attempt to produce test animals that avoid all sources of artefact from the ML selection regime, including the reversed inheritance of sex chromosomes, the foreign cytoplasm, the maternally inherited Y chromosome, and the translocated autosomes. To do this, we used males and females produced from the second recombination cross in the ML treatment. Through many generations of backcrossing of ML genetic content into a control derived line, we were able to produce RC2 flies carrying ML genetic content without the associated artefacts. To create the actual test animals, we used a crossing design between RC2 and MC flies.

From a full factorial crossing design, 4 types of male offspring and 3 types of female offspring can be obtained (fig A2a). From crosses of MC males and females, we derive our control treatment of test animals (male and female), also labelled MC. From RC2 males and females, we can derive flies carrying completely ML type genomes, labelled ML_{DD} (ML

double dose). Both directions of the hybrid cross produce identical females, carrying an ML haploid genome. These, like males carrying a single complete haploid genome from the ML treatment are labelled ML_{SD} (ML single dose). While crosses with MC sires and RC2 dams produce ML_{SD} males, crosses between RC2 sires and MC dams produce males carrying an X chromosome from MC populations and a single dose of ML autosomes. These males are labelled $ML_{SD(a)}$. Crossing designs used were not fully factorial in all the experiments reported below, but the labelling scheme indicates the crosses carried out as described here. The different test animals studied were denoted using the variable 'Treatment' in analysis.

While fitness assays were conducted using test animals as described above, a slightly different design was used to produce test animals for mate choice, sperm competition, body size and development time assays (fig A2b). Here, test animals were produced using a cross analogous to the second recombination cross used for generating RC2 males to create both ML and MC test flies. First, ML and MC male haplotypes are captured using CG (bw^D) females. Sons from these crosses carrying the haplotypes are crossed to virgin MC or RC2 females to produce test flies. Here too, as before, the treatments are labelled as per the system above (MC, ML_{DD} , ML_{SD} , $ML_{SD(a)}$). While mostly identical to the design used for fitness assays, the male test animals from this design carry Y chromosomes from clone generator lines that may have been exposed to feminization or mutation accumulation effects. Due to this oversight, it is possible that there are effects in the phenotypes displayed by the test animals in response to the Y chromosome.

To study genetic variance for fitness in these populations, we created hemiclinal lines (as described in Chippindale *et al.* 2001) using single haploid ML-1 and MC-1 genomes sampled through crosses with CG females. Each individual haplotype was amplified entirely in sons using crosses with CG (bw^D) females; and had its X chromosome passaged to a daughter using crosses with a female carrying MC derived autosomes and balancer X

chromosomes (fig A3). These sons and daughters respectively were then crossed to one another to produce test animals carrying ML and MC haplotypes, along with control derived autosomes. Females derived were homozygous for the X chromosome, and males carried the Y chromosome from the CG (bw^D), much like the test animals from the mate choice and sperm competition assays.

Across all assays, test animals were maintained at a larval density of 80-100 animals per vial. Virgin flies were collected 6-8 hrs from eclosion using light anaesthesia between days 8-10 PO. Non virgin test animals were collected on days 10-11 PO.

Fitness, mate choice and sperm competition assays required the use of phenotypically marked competitors. These lines allow us to identify successful mates and measure competitive siring/damming success by distinguishing the progeny phenotype. To this end, we backcross a recessive eye colour markers into control derived lines to create these competitor populations. Two such populations are created, combining flies across the different replicate populations of the MCs. In the first, we introduce the pink-peach (p^p) eye colour marker, and in the other we introduce the brown (bw^l) eye colour marker. Collectively, we refer to these lines as recessive competitors – “Cr”.

Assays

Competitive Reproductive Fitness (CRF) assays

CRF for was assayed for both males and females. Following the crossing design, test females were collected as virgins, and test males were collected on day 10-11. On day 12, 30 test animals were combined with 30 Cr individuals of the same sex, and 50 Cr of the opposite sex (both carrying the recessive eye colour marker). These animals were held together in mini cages with ad libitum yeast between days 12-14 PO, to mimic the reproductive conditions of the selection treatment. For each treatment (within each replicate pair of lines), we assessed 6

such competition arenas (labelled contests in analysis), and collected 6 vials of eggs to measure CRF.

Male CRF was assayed thrice, in generations 50, 64 & 70 of the selection experiment. In generations 50 and 64, only ML_{SD} and MC animals were tested. All four were tested in generation 70. For the experiment conducted in the 70th generation, males were collected as virgins. Female CRF was assayed twice, in generations 48 & 50 of the selection experiment. On both occasions, we only tested MLSD and MC animals.

Hemiclonal analysis of fitness

Conducted in generation 78, CRF assays for hemiclonal analysis were designed very similarly to the CRF assays described above for males. The competition size was reduced in response to the increase in number of lines being studied. Here, we combined 4 test males with 4 Cr males and 6 Cr females. For female fitness, 4 virgin test animals were combined with 3 Cr males. Rather than competitive reproductive fitness, female productivity was assayed as a proxy for fitness. In both sexes, up to 5 such contests were set up for each line, depending on the availability of usable test animals. Complete hemiclonal data for both sexes was collected from 76 lines out of an initially sampled 80.

Mating success assays

We assayed female mate choice (or male mating success) in generation 66, using progeny eye colour. Progeny were also sexed and enumerated to test for fecundity induction and sex ratio drive effects. Following the crossing design, test males were collected on day 10-11. On day 12, single test males were combined with a Cr male, and a Cr virgin female. When one of the two possible pairs begin copulation, the excess male was aspirated out by gently removing the foam plug without disturbing the pair in copula. After the mating ended and animals separated, the remaining male was also removed, and the female was left

undisturbed to oviposit for 48 hours under *ad-libitum* yeast conditions. The time of introduction (observation start time), amplexus formation (mating start time) and separation (mating end time) were noted, which provides us measures of mating latency and mating duration. The offspring sired in each vial were sexed and enumerated. The eye colour of the offspring in each vial was noted to identify the sire that successfully mated. For each treatment (within each replicate pair of lines), we assessed 50 such competition vials.

Sperm offense assays

Sperm offense (or P2) was assayed (in generation 74) by serially mating Cr females first to Cr males and subsequently to target males. Following the crossing design, test males are collected on day 10-11. On day 12, 12 virgin Cr females with *ad-libitum* yeast nutrition are combined in vials with males from the same population. Matings were observed carefully until the point that synchronous observations were possible (if some matings end before other matings begin, it is not possible to identify if a single female was mated twice, or if all the females were mated singly. Although unlikely, as recently mated females tend to be unreceptive, it is possible that this system allows for some females to be multiply mated. This is however consistent across all the treatments). In all vials, at least all but 2 females were directly observed to have been mated. Using a treatment where no second male was provided, we found that this system resulted in 97% fertile females. An hour after synchronous matings ended, the flies were gently anaesthetized with CO₂, and males were discarded. On day 13, 10 test males were combined with females. Second matings were observed carefully to ensure that no single female was multiply mated at this stage. To achieve this, we observe all the vials to note when matings began in these vials and continue observing the vials for a period of ~3 hours after this point to note the number of matings seen, at the end of which the males were discarded to ensure that the first mated female does not return to a receptive state. We then randomly selected 10 females and transferred them to individual test tubes with *ad-*

libitum yeast to oviposit for 24 hours, after which they were discarded. Due to the arbitrary cut-off in the second mating period, dictated by the first pair to copulate in each vial, we do not compare the number of matings achieved by target males in each treatment, instead assessing the proportion of offspring sired where a second mating has successfully taken place. Offspring from each tube were phenotyped by eye colour and enumerated. For each treatment (within each replicate pair of lines), we assessed 30 such competition vials.

Statistical Analysis

Statistical analysis was conducted in R version 4.2.3 -- "Shortstop Beagle" (R Core Team 2023). Data was visually assessed for residual normality and using Bartlett's test for residual heterogeneity. Where generalised linear models were used, residual dispersion was tested using the DHARMA package (Hartig 2022).

Linear mixed models were employed to analyse CRF. Treatment and replicate population were used as fixed factors in the analysis to explain the proportion of offspring sired/dammed by the target individuals. To avoid pseudo-replication, contest was included as a random factor. Where significant differences were identified, we employed planned contrasts between the treatments in a single replicate population pair with a corrected alpha value.

In the hemiclinal analysis of fitness, we analysed genetic variance for fitness in each selection regime, for each sex, using a random effects model that explained the proportion of offspring sired / dammed by the target individuals, using the hemiclinal line as a random factor. The proportion of variance explained by the random factor line was then compared between selection treatments using an F test. Fitness data from the hemiclinal analysis was also analysed using a mixed model, where selection was used as a fixed factor. Here, we did not include replicate as in the case of CRF, as the study was conducted only on replicate population pair 1. Here too, line was included as a random factor. Additionally, mean

estimates of male fitness and female fitness for each line from each selection regime were analysed using a Pearson correlation test to test if the ML population displayed a different intersex genetic correlation for fitness from the control. The correlation coefficients were compared against one another using a Fisher's Z transformation.

To study the mating success of target males in female mate choice trials, we used a generalized linear model with a binomial distribution. The proportion of mating successes for target animals was explained using selection and replicate as fixed factors. Likewise, log(mating latency), mating duration, fecundity induction (offspring count) and sex drive (offspring sex ratio) were analysed using a Gaussian distribution for tubes, with selection and replicate as fixed factors.

For sperm offense, the proportion of offspring sired by the second male was analysed in two steps, as if in a Hurdle model. First, to create a data transformation that enabled the analysis, we subtracted the proportion of offspring sired from 1. This inverted measure was then separated in two parts – values of zero (where all the offspring are sired by the target male) and non-zero values. The number of zeroes attributable to each treatment was analysed as a generalized linear model with a binomial distribution, using treatment and replicate as fixed factors.

RESULTS

Competitive reproductive fitness (CRF)

Male fitness

In generations 50 and 64, we found no effect of treatment, replicate or their interaction on male CRF (table A1a). In generation 50, ML_{SD} displayed a CRF of 0.3399 +/- 0.0125 (mean +/- 1.96*se) compared to 0.3215 +/- 0.0149 for MC (fig 2.1a). In generation 64, these figures were 0.3406 +/- 0.0178 and 0.3222 +/- 0.0171 respectively (fig 2.1b).

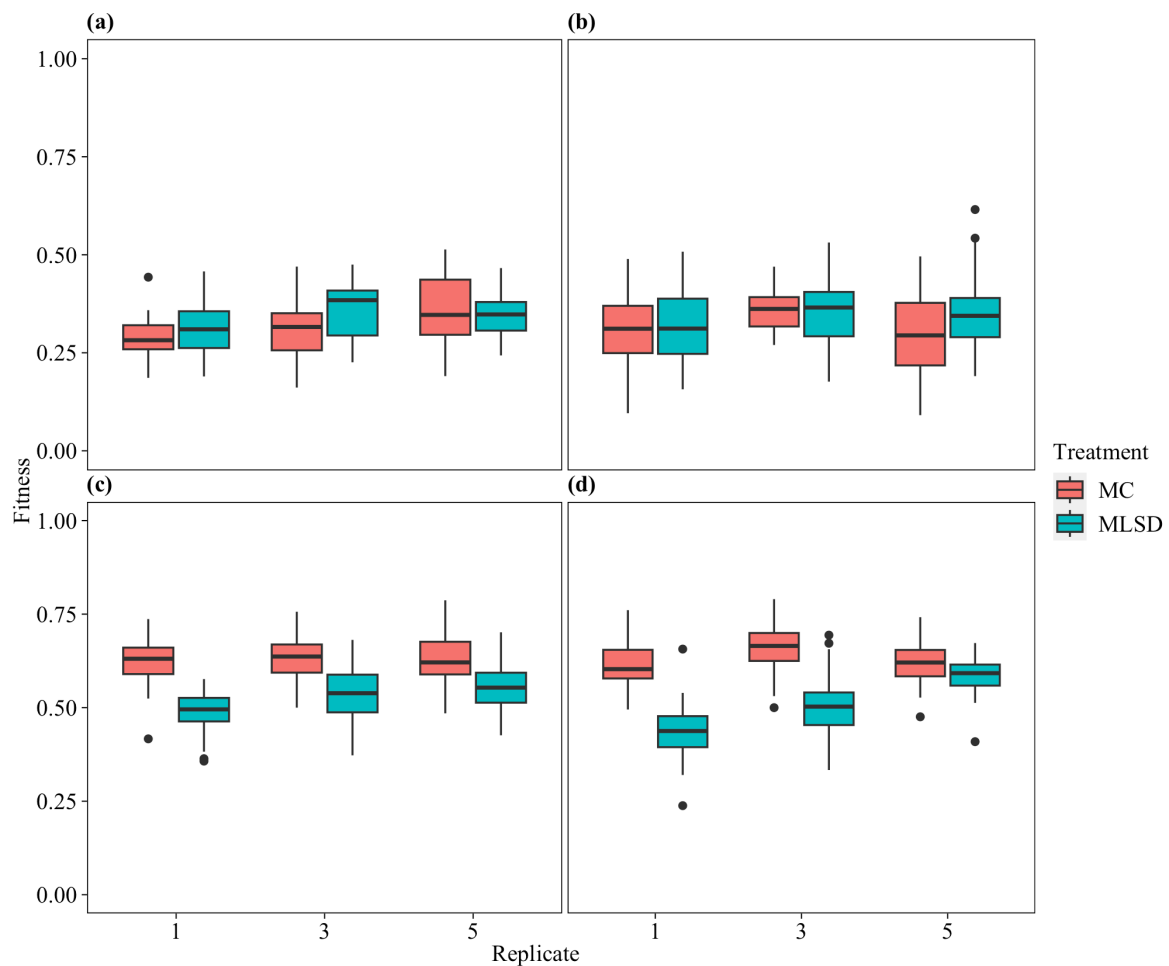


Figure 2.1. The proportion of offspring (CRF) of target MC (red) & ML_{SD} (blue) males in (a) generation 50, and (b) 64; target females in (c) generation 48, and (d) 50. ML lines were tested as whole (X, II, III) haplotypes in a single copy

In generation 70, we found a significant effect of treatment on reproductive fitness ($p < 0.001$), and no effect of replicate or their interaction term (although the interaction term approached the $\alpha = 0.05$ threshold) (table A1b). Excluding replicate (and the corresponding interaction term), we conduct pairwise contrasts between all 4 treatments to find that the only significantly different pairs were ML_{SD} - ML_{DD} (Tukey adjusted $p = 0.006$) and MC - ML_{DD} (Tukey adjusted $p = 0.01$). CRF for each treatment (fig 2.2) was as follows: MC (0.4508 ± 0.0183), ML_{SD} (0.4730 ± 0.0172), $ML_{SD(a)}$ (0.4172 ± 0.0176), ML_{DD} (0.3719 ± 0.0158).

Female fitness

In generation 48 we found a significant effect of selection treatment ($p < 0.0001$) on CRF, and no effect of replicate or their interaction term on CRF (table A3a). ML_{SD} females displayed a CRF of 0.5269 ± 0.0132 , and MC females 0.6280 ± 0.0120 (fig 2.1c).

In generation 50, we found a significant effect of selection treatment ($p < 0.0001$), replicate ($p < 0.0001$) and the interaction term ($p < 0.001$) on CRF (table A3a). Here too, ML_{SD} females displayed reduced CRF compared to MC animals. Contrasts between treatments within each replicate revealed that the difference between ML_{SD} and MC females of replicate 5 was not significant ($p = 0.074$) at the adjusted $\alpha = 0.017$ threshold for 3 comparisons. Here too, the ML_{SD} mean was found to be lower than that of MC . Overall, ML females displayed a CRF of 0.5072 ± 0.0189 , and MC females 0.6308 ± 0.0120 (fig 2.1d).

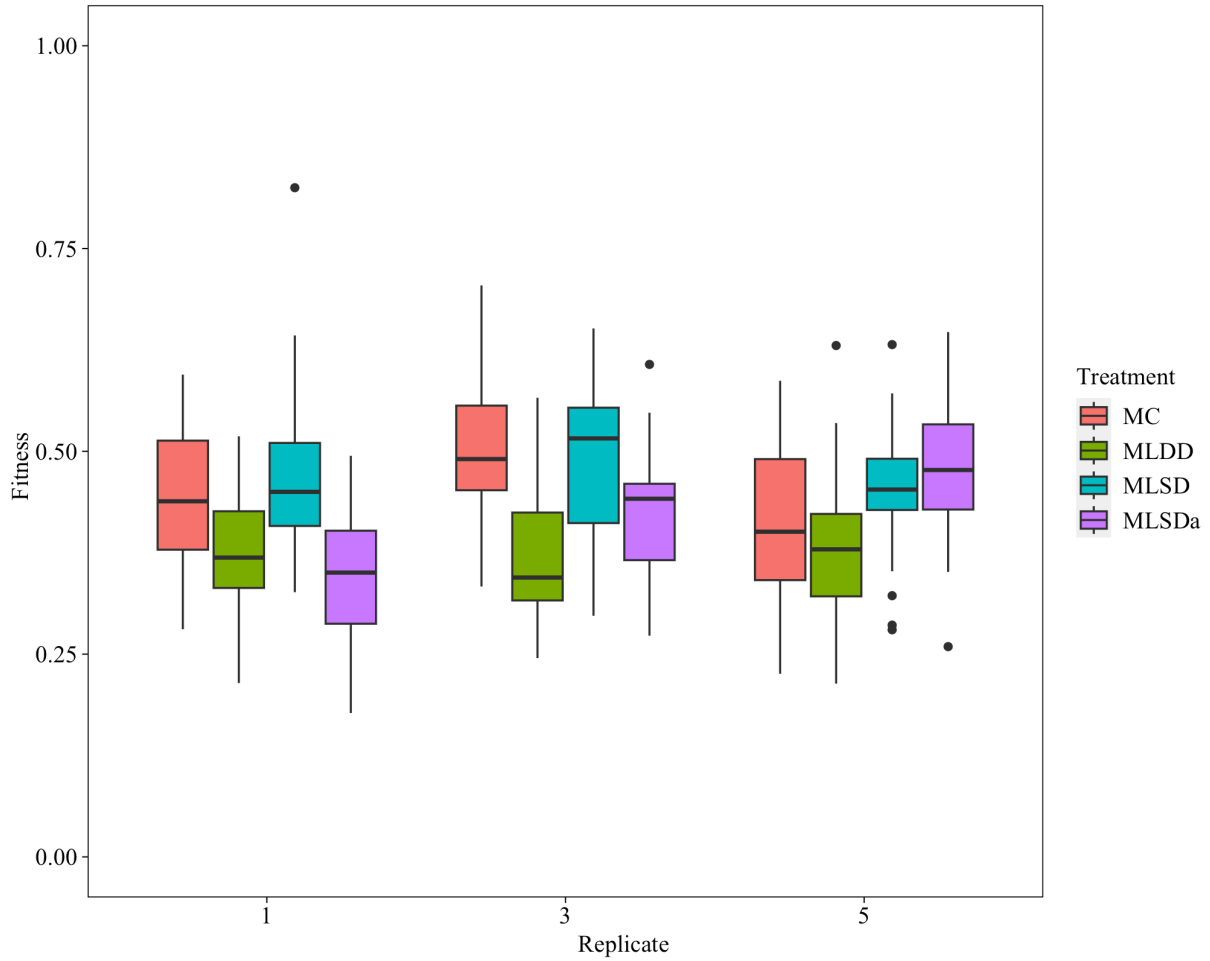


Figure 2.2. The proportion of offspring sired by target males in generation 70. ML lines were tested as whole (*X, II, III*) haplotypes in a single copy (ML_{SD} - blue), with just the autosomes in single copy ($ML_{SD(a)}$ - purple) or with all autosomes from the ML populations, a “double-dose” (ML_{DD} - green); see text for details.

Hemiclonal analysis of fitness

Male fitness

We quantified genetic variance for fitness for both ML (0.0509) and MC (0.1771) males as the proportion of variance attributed to ‘line’ in a random effects model. Using an $F(37, 37)$ test, we found that these variances were significantly different from one another ($p = 0.0001$) (table A2a). Male fitness analysed using selection as a fixed factor and line as a random factor revealed an effect of selection ($p < 0.0001$) (table A2c). This model did violate the Bartlett’s test for homogeneity of variances, but as shown above, that is likely a feature rather than a bug here. Overall, ML males displayed a CRF of 0.553 ± 0.0255 , compared to MC males 0.455 ± 0.0385 (fig 2.3).

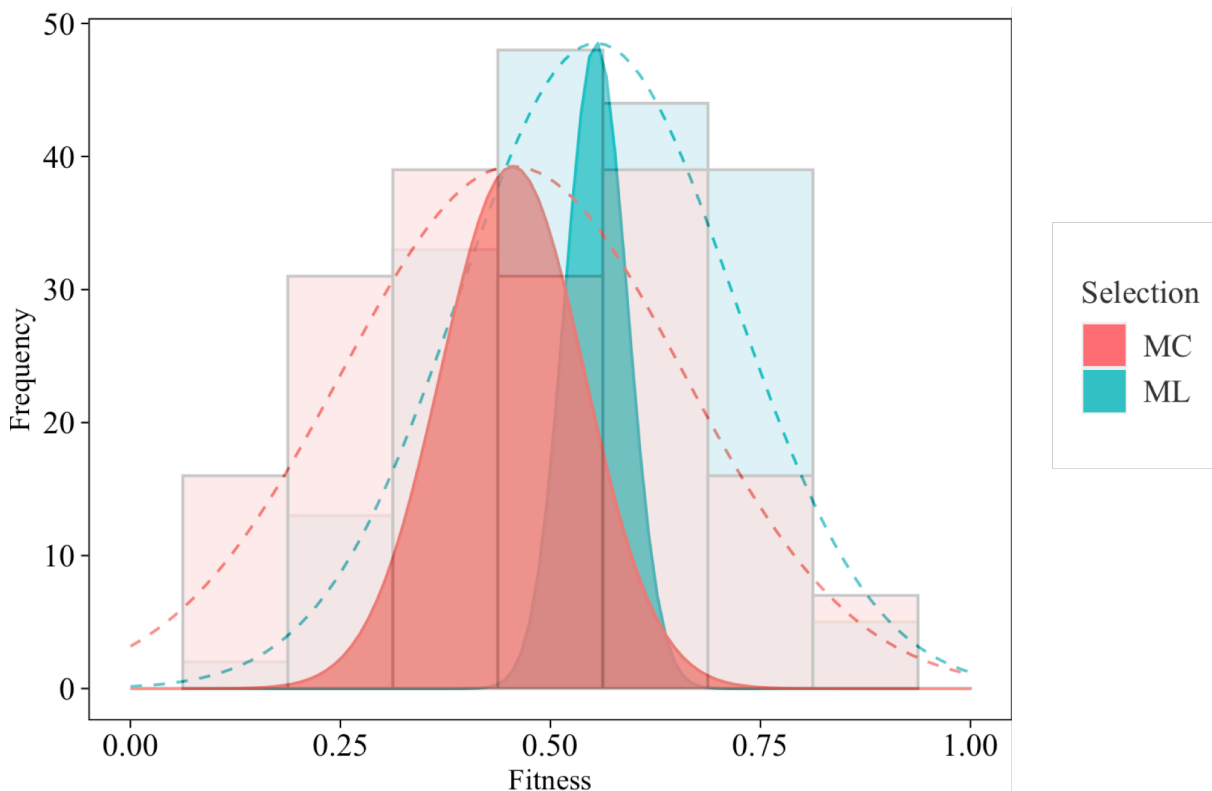


Figure 2.3. Heritable variance for male CRF. Histograms represent actual measurements from hemiclonal lines from ML (blue) & MC (red) treatments. Mean, variance for each treatment is depicted using dotted curves. Solid distributions depict heritable variance.

Female productivity

Likewise, we quantified genetic variance for productivity in ML (0.467) and MC (0.424) females. These were not significantly different from one another in a F(37,37) test ($p = 0.39$) (table A2b). Female productivity analysed using selection as a fixed factor and line as a random factor revealed no effect of selection ($p = 0.29$) (table A2c). ML dam productivity was 17.69 ± 2.049 and for MCs it was 16.09 ± 1.915 .

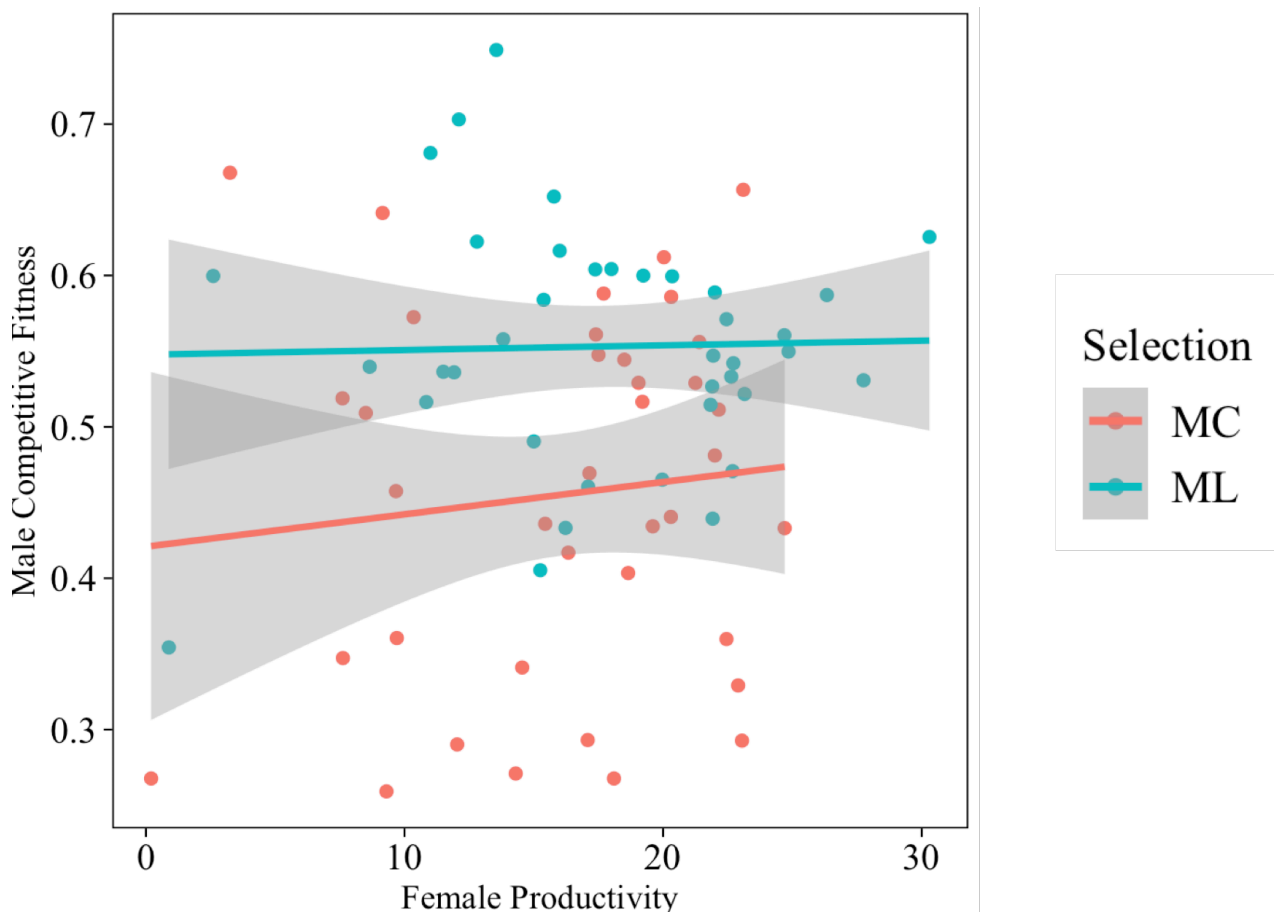


Figure 2.4. Intersex genetic correlations plotted between female productivity and male CRF for each hemiclinal line from ML (blue) and MC (red) treatments. A linear regression is plotted for each treatment. Shaded region depicts 95% confidence interval.

Intersex genetic correlation for fitness

Intersex genetic correlations for fitness for both the ML ($\rho = 0.0247$) and MC ($\rho = 0.11$) population were not significantly different from 0 (table A2d). Likewise, they were not significantly different from one another ($p = 0.34$) (table A2e).

Mating success

Mating success, latency and duration

We find no effect of selection, replicate or their interaction upon the male chosen for mating, mating latency or mating duration (tables A4a, b, c). Across all replicates however, we note a consistent direction of difference in mate choice success. ML males were more successful, with greater differences in replicates 1 (61.7% success for ML vs 50.0% MC) and 3 (56.5% vs 46.5%), but also in 5 (44.7% vs 40.0%) (fig 2.5a). It is possible that there exists an effect the study was not sufficiently powered to capture. MLs on average showed a mating latency of 13.96 +/- 4.14 mins and a mating duration of 18.43 +/- 1.00 mins. For the MCs, these figures were 14.93 +/- 4.33 mins and 17.95 +/- 1.48 mins respectively (fig 2.6a, b).

The model analysing mating duration deviated from homogeneity of variances as tested by Bartlett's test, suggesting a potentially flawed analysis. However, from data visualization it appears that there are no real differences in this dataset, as in the model.

Fecundity induction, sex ratio drivers

We find no effect of selection, replicate or their interaction on fecundity induction, or sex ratio drivers (tables A4d, e). Sex ratio for all the populations hovered around the expected 0.5 mark. ML male offspring were at 0.507 +/- 0.017 and for MCs it was 0.503 +/- 0.028 (fig 2.6c). ML males induced a fecundity of 51.09 +/- 2.94 offspring in partners, while MC males achieved 50.94 +/- 3.21 (fig 2.6d).

Sperm offense

We find no effect of selection treatment, replicate or their interaction on either of the sequential steps of analysis on sperm competition (tables A5a, b). Proportion of offspring sired by each treatment as a second male was as follows: MC (0.8985 \pm 0.0271), ML_{DD} (0.8474 \pm 0.0353), ML_{SD(a)} (0.8855 \pm 0.0299), and ML_{SD} (0.9034 \pm 0.254) (fig 2.5b).

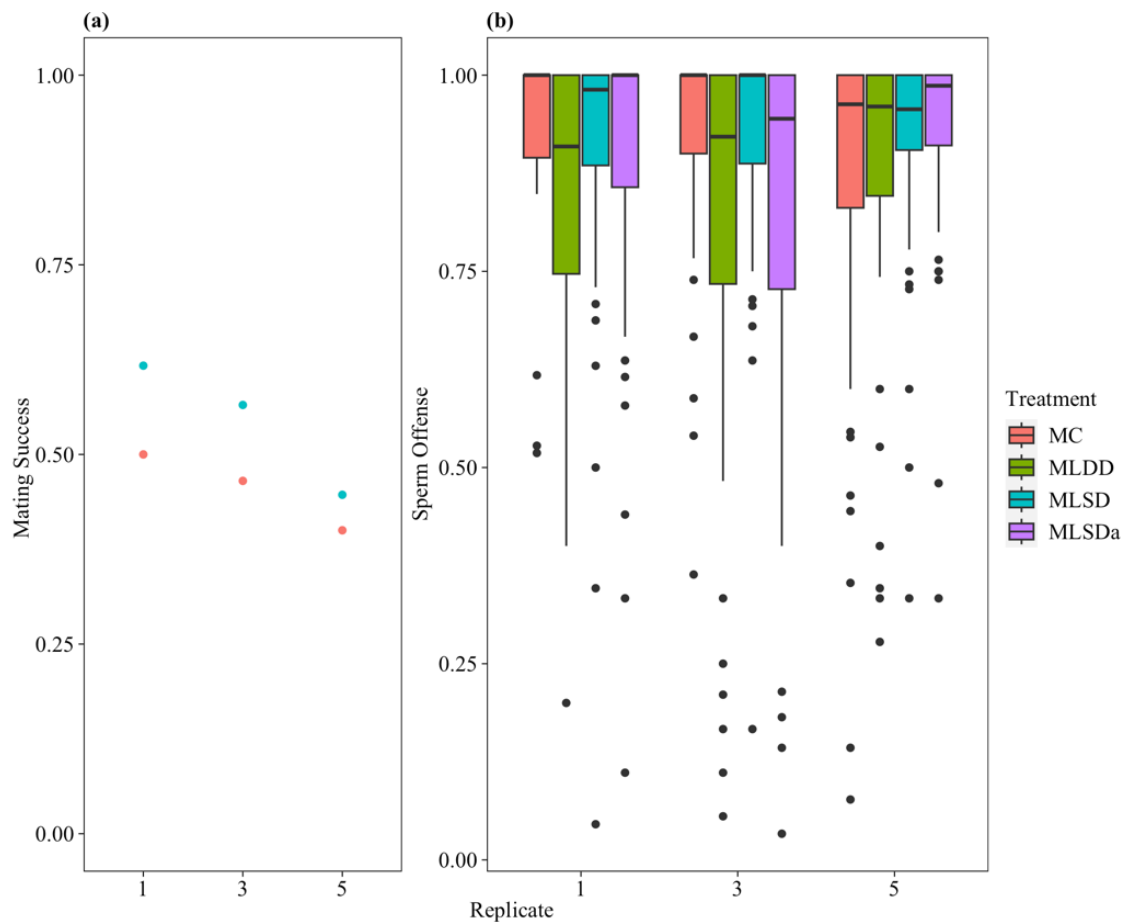


Figure 2.5. (a) The proportion of target MLSD (blue) or MC (red) males gaining mating with females relative to a marked competitor line and (b) post-copulatory reproductive success as second of two mates (i.e., P2 or sperm offence). ML lines were tested as whole (X, II, III) haplotypes in a single copy (ML_{SD} - blue), with just the autosomes in single copy (ML_{SD(a)} - purple) or with all autosomes from the ML populations, a “double-dose” (ML_{DD} - green); see text for details.

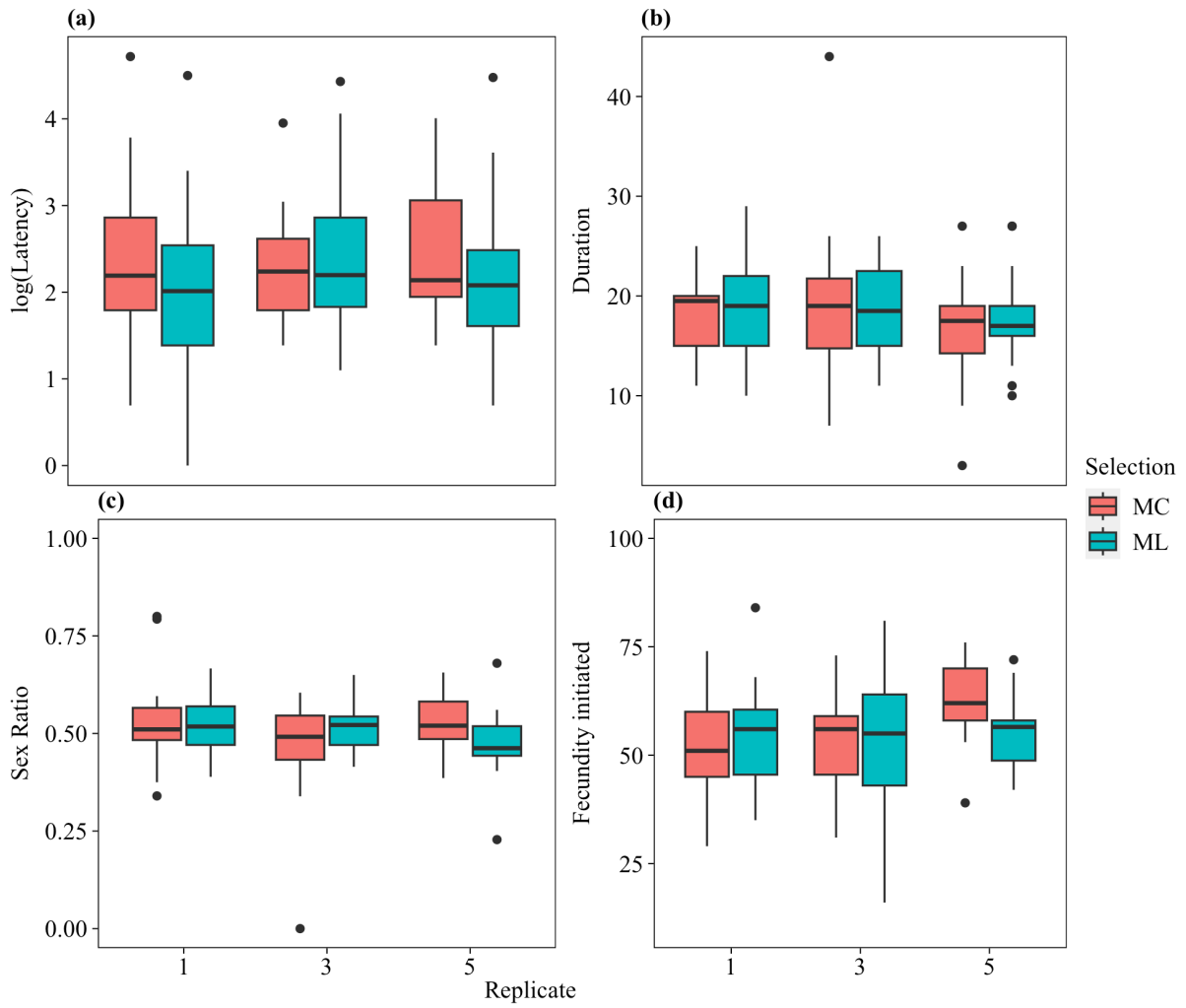


Figure 2.6. Measures of (a) latency to mating (log transformed), (b) duration of matings, (c) brood sex ratios and (d) fecundity induction for target ML_{SD} (blue) and MC (red) males.

DISCUSSION

Sex limited evolution has been a powerful tool in the study of IaSC. Our study fails to replicate a key proof of principle prediction of this system, the generic improvement of male fitness in haplotypes under male limited selection tested in ancestral conditions. We find no differences in male reproductive fitness or components thereof – namely mating success, fecundity induction and sperm offense. By contrast, we find that female fitness of these haplotypes declines relative to controls as predicted by sexually antagonistic variation. Through a hemiclinal analysis of genetic variance, we demonstrate a significant reduction in heritable variation for male fitness in one selected population relative to its paired control, but in the presence of a potential artefact. We found no change in heritable variation for female fitness, or in the intersex genetic correlation for fitness between selection treatments.

Across three assays of male reproductive fitness, with three replicate pairs each, we found no differences between male limited animals and their matched controls, except for ML males carrying a double dose of ML autosomes being worse than ML males with a single dose. The absence of response to ML selection might be explained by four possibilities. (1) First, if there was no SA genetic variance for fitness in the source populations utilized due to fixation of alleles in loci critical to fitness, selection would not be able to act upon these populations until novel male benefit mutations occur in the population. The base populations used to derive the selection experiment are long domesticated animals, creating the possibility for such fixation events. (2) Alternatively, intralocus sexual conflict is not as pervasive as previously believed in *Drosophila melanogaster* and needs to be studied across more base populations. (3) Third, it is possible (although unlikely) that selected haplotypes are recessive when paired with control haplotypes in males. Specifically, this would require no evolved changes in the selected Xs (haploid as it is in males), combined with recessivity in selected autosomes. (4) Lastly, the adaptive response in these populations could be restricted

to the specific selection conditions imposed by the breeding design used. For instance, haplotypes may evolve to be locally adapted to the females used, or in concert with the genetic background they are expressed in. This could also be understood as selection to counter fitness costs imposed by selection artefacts.

We explore these ideas in detail in sibling manuscripts (Thyagarajan *et al.*^b (chapter 3), Thyagarajan *et al.*^c (in prep)). Briefly, we find that the selection lines do adapt locally in response to multiple selection artefacts. We find evidence that these populations still have abundant genetic variance to display adaptive responses, and strong evidence against the recessive haplotype hypothesis. Given that we find artefactual adaptation, we are unable to definitively assess the presence/absence of IaSC using this selection experiment.

If the evolved response is dominated by compensatory adaptation to artefacts, the lack of male fitness improvement under the test conditions sans artefact can come about through 2 routes - through local adaptation to selection conditions that (1) does or (2) does not impose a cost upon fitness under ancestral conditions. While scenario (2) is directly in agreement with the results described herein, scenario (1) requires us to explain why there is no difference in male fitness in our assays. To explain this, we note that it remains possible (although not parsimonious) that the selection experiment simultaneously results in a generic improvement in male fitness as predicted, combined with a reduction in fitness in testing conditions, relative to locally adapted controls.

In keeping with predictions, ML female fitness was found to be lower than MC female fitness. In previous studies, the decline in female fitness of ML selected haplotypes, combined with the masculinization of phenotype was treated as clinching evidence of IaSC. In the context of the results in this study however, it must be noted that this could simply be due to a relaxation of selection acting upon female-limited loci of the genome. Alternatively, such a consistent reduction in female fitness might also come about from sexually

antagonistic costs. Here, SA fitness costs experienced by selected haplotypes in females may be consistent across local and ancestral conditions, while male benefit variation that is specific to the local conditions of selection.

Besides these curious results, we were also initially surprised to find low male CRF across MLs and MCs ($\sim 1/3^{\text{rd}}$) when the number of target males and competitors in competition arenas were the same (similar female assays showed CRF values greater than 50%). This led us to wonder if there was an effect of female preference for conspecific males in the competitor populations used. We also noted that competitor males were being collected as virgins along with their females for convenience, unlike the target males. Using virgin target males in the third assay in generation 70, we found higher CRF values around 0.45-0.47, suggesting that male status did play a role. However, it is possible that a conspecific preference also exists, given that the values still did not hit 50%.

For the remainder of results described, the target males of all treatments include a CG derived Y chromosome. We found a suggestive (but not statistically significant) trend of ML males gaining more success in mating trials, but no differences in latency to mating, duration of mating, fecundity induced, offspring sex ratio, or sperm offense.

We cautiously interpret these results to be in agreement with our primary finding of no male fitness differences between selected and control animals. While the analysis of mating trials shows no effect of selection on mating success, we note that this could be due to the limited sample size in the context of a binary variable.

Previous experiments are equivocal about the evolution of sperm competition traits in response to male-limited selection. Rice (1996), (1998) found selected animals to have evolved in a greater sperm offense capability against both CG and non-CG females, and improved sperm defense, but only with CG females. Working with the same base population, Jiang *et al.* (2011) found no difference between selected and control males in terms of sperm

offense or defense, against either female type. Here, we find no improvement in sperm offense in selected animals, against non-CG females. We interpret this as a generic lack of improvement in sperm offense, independent of artefacts.

In the hemiclinal analysis, we identified that genetic variance for male fitness was lower for ML_1 compared to MC_1 as predicted. Genetic variance for female productivity was not different between ML_1 and MC_1 . However, we also found that male ML_1 fitness was greater than male MC_1 fitness, and female ML_1 productivity was not different to MC_1 productivity, in seeming contradiction with the CRF results. First, we note that for logistical ease we chose to assay female productivity here, rather than CRF. Unlike CRF, productivity as assayed does not depend on sexual selection or inter-locus sexual conflict (IeSC), which might explain the absence of difference identified. For male fitness, we interpret this difference in male CRF between the two populations as being a response to CG derived Y chromosome. Therefore, we do not feel confident in interpreting this reduction in genetic variance for male fitness in MLs as a response to the removal of balancing selection. It could simply be inflated genetic variance for fitness in the MCs that are exposed to a novel selection artefact resulting in more variable fitness outcomes. Another point of interest was the difference in genetic variance estimates for CRF in males and productivity in females. The heritable variation for female productivity being much higher could be because of stronger sexual selection on males. However, this may also be a consequence of productivity being assayed in females rather than CRF.

Overall, we find that more than 80 generations of male limited selection do not result in a phenotype of increased male fitness when these genomes are expressed in the absence of artefacts associated with selection. In previous iterations of male limited selection experiments, such fitness effects have been identified consistently, using a different source population (LH_M), and in the presence of selection artefacts as in Rice (1996) (fitness tested

under selection conditions), Rice (1998) (CG derived Y chromosomes, double dose haplotypes), Prasad *et al.* (2007) (translocated autosomes, CG derived Y chromosomes and CG females) and Abbott *et al.* (2013) (CG derived cytotypes, Y chromosomes and reversed sex chromosome inheritance). Our results suggest the need to generalise these findings across more baseline populations, with improved controls to account for potential artefacts.

CHAPTER 3

Compensatory evolution dominates the response in a sex-limited selection experiment in *Drosophila melanogaster*.

ABSTRACT

The conflict of opposing selection forces acting on traits with correlated expression between the sexes is labelled intralocus sexual conflict (IaSC). We previously reported on a male limited selection experiment designed to investigate IaSC through a sex-limited evolution experiment that resolved it in favour of males. This selection experiment did not improve male fitness as predicted, suggesting either the absence of sexually antagonistic variance to select upon, or a role for adaptations to selection artefacts in the design. In this follow up, we report on the fitness effects of these selection artefacts. Firstly, we address the consequences of a foreign cytoplasm, a female-exposed Y chromosome, translocated autosomes, and the combined effect of these artefacts. Selected lines consistently show a substantial (66%) improvement in male fitness under conditions including all the genetic artefacts. We find that the (replicate) selected lines respond differently to various artefacts in the genetic background expressed individually; and demonstrate significant improvements in fitness in response to the foreign cytotypic and female-exposed Y chromosome. Second, we investigate the consequences of male adaptations to the evolutionarily arrested females used to achieve the ML protocol. On its own, we are unable to detect an effect of female arrest, but we find a strong interaction effect: males reared as per the selection protocol display a huge (>100%) improvement when tested with the females used in the selection experiment. Additionally, we find that male adaptation to the foreign cytotypic produces a correlated fitness improvement when selected haplotypes are expressed in females.

INTRODUCTION

Anisogamous organisms encounter a fitness load from the impact of sex-specific selective pressures on shared traits. This phenomenon is termed intralocus sexual conflict (IaSC). Given the widespread existence of sex-specific selection pressures and positive genetic correlations in trait expression between sexes, IaSC is anticipated to be pervasive in the evolutionary dynamics of gonochoric populations. Specifically, it should influence: (1) the preservation of genetic variance for fitness via balancing selection, (2) the evolution of trait dimorphism, and (3) patterns of sex-biased gene expression and asymmetric selection on male and female-biased genes.

In a previous study (Thyagarajan *et al.*^a (chapter 2)), we describe a sex-limited selection experiment aimed at subjecting haplotypes of *Drosophila melanogaster* to selection for male fitness, while circumventing opposing selection pressures on female fitness. We predicted an enhancement in male fitness within the selected lines, achieved at the expense of female fitness due to the fixation of male benefit alleles at SA loci. In contrast with previous studies employing this technique, we found no increase in male fitness of haplotypes subject to male-limited (ML) selection relative to their matched controls (MC). Males from ML lines do not outperform controls in mate choice trials, fecundity induction or sperm offense.

This lack of evolved response in male fitness could be due to an absence of sexually antagonistic (SA) variation in the populations used to initiate the selection experiment. It is possible that the populations intrinsically harbour limited SA variation compared to the populations previously studied in this context. Previously, working with ancestors to the populations used in this study, Mallet *et al.* (2011) found a non-negative intersex genetic correlation for reproductive fitness ($r_{w,g,m-f}$), which would be consistent with no IaSC although not diagnostic (Connallon & Matthews, 2019). IaSC might also be diminished by reduced

genetic variance. For instance, long term domestication under stable conditions could result in gradual fixation even in loci that typically house balanced polymorphisms, resulting in reduced genetic variance and IaSC. Previous work conducted with the source populations shows some signs of reduced genetic variance, seen in the form of a trend of hybrid vigour in juvenile survival (Robinson *et al.* 2023).

Alternatively, artefactual sources of selection in the evolution experiment could have resulted in adaptive responses to specific conditions. The ML evolution experiment depends upon a breeding design that uses “clone generator” (CG) females, derived each generation from an independent stock population. The use of these females introduces two modes of selection unrelated to sex-limitation. First, the independent origin of the females “arrests” them, preventing co-evolution with their ML mates (Rice 1996). By contrast, the ML males can potentially adapt to the females, without ensuing counteradaptations. Second, the genetic background that the ML haploid genomes evolve in is both arrested, and far removed from that of their source population. Adaptation to selection from either of these sources could be specific to the selection conditions, without improving male fitness under generic test conditions. Further, such adaptation could take place through selection for male benefit but sexually antagonistic alleles, male benefit alleles that are neutral to female fitness, or alleles that produce correlated fitness benefits in both males and females.

Sexually selected traits may be subject to runaway coevolutionary dynamics and have the potential to diverge rapidly between populations. This divergence between signal-receiver traits may take arbitrary trajectories between populations in allopatry. These include pre-copulatory traits such as courtship, and post-copulatory traits like ejaculate composition, morphology, seminal proteins, and mate harm. Given the diverged evolutionary history of the CG and ML source populations, the CG females likely represent a novel (but static/arrested) landscape of sexual selection for the ML males. Besides a long diverged evolutionary history,

the CG females also carry substantial deleterious mutations that make them very different from wild type *D. melanogaster* flies. These flies display poor motility, diminished vision, reduced fecundity, reduced resistance to mating attempts and rapid mortality (when mated) relative to control derived females. Previous ML evolution experiments have consistently found an increase in male fitness in selected lines when fitness is assayed using CG females (Rice 1996, Prasad *et al.* 2007). While Rice (1996) finds improved sperm competitive ability, Jiang *et al.* (2011) (using the same populations as Prasad *et al.*) find no evolved response in sperm competition. Rice (1996) also finds increased levels of mate harm by the selected lines.

The CG females are also responsible for supplying a genetic background for the haplotypes under ML selection. First, this includes a translocated II-III autosome pair that prevents independent assortment of the autosomes through aneuploid mortality. Along with the translocation mutations, the autosome pair carries multiple visible recessive markers for identification. Previous studies assume that the lack of visible mutant phenotype in the selected ML animals implies a general recessive nature of alleles on these autosomes, without explicitly testing for associated fitness consequences (Rice 1996, Prasad *et al.* 2007).

The X chromosome is hypothesised to be a hotspot for sexually antagonistic variation (Rice 1984, although see Kidwell *et al.* 1977). The inclusion of the X chromosome in the ML selection experiment is made possible by the compound-X chromosome pair (XXY karyotype) of the CG females. These females produce 2 types of gametes – eggs carrying a compound pair of X chromosomes that can result in viable XXY zygotes like the dam, or eggs carrying a Y chromosome that can result in XY males when fertilized by X carrying sperm. Using these Y carrying eggs, we reverse the parent of origin of the sex chromosomes in male offspring. Given that X carrying sperm normally produces daughters, it is possible that males undergo selection to epigenetically modify their X chromosomes in favour of their daughters. In the ML selection experiment, this selection pressure is reversed, potentially

resulting in fitness effects specific to the conditions of reversed sex chromosome inheritance, and X chromosome drive through male-biased sex ratios. (explicit description of paternal X transmission)

A second artefact of the XXY karyotype is the CG Y chromosome. As a sex limited (non-recombining) chromosome, the Y has a limited number of genes. Despite this degeneracy, the Y is found to play an important role in male function through the regulation of gene expression on other chromosomes (Chippindale & Rice 2001, Lemos *et al.* 2008, Jiang *et al.* 2010). In these studies, unrelated Y chromosomes were expressed in a common genetic background, resulting in significant differences in gene expression, especially in loci displaying male-biased gene expression. The use of the foreign CG Y chromosome is likely to cause disruptions in gene expression for the haplotypes undergoing selection. Moreover, the CG-Y is unique – it spends alternate generations in males and females, undergoing selection as usual in male flies, but experiencing either relaxed selection or female-specific selection gradients when expressed in females. Branco *et al.* (2017) study a similar line of *D. melanogaster* and show that an XXY karyotype results in changes in the expression of genes expressed in the ovaries, mitochondria, and larval neuronal tissues relating to mating and reproductive behaviour, suggesting that Y chromosomes in XXY females likely undergo selection to benefit female function.

Lastly, the use of the CG females also introduces a foreign cytotype. The evolution of uniparental cytoplasmic transmission is thought to originate from the selective pressures imposed by selfish mitochondrial (mt) genotypes. However, the maternal transmission of cytoplasm results in an evolutionary dead end for mtDNA in males. The mitochondrial genome's interests are exclusively dependent on the female: natural selection favours female-benefit variants, even when harmful towards male function (Cosmides and Tooby 1980, Frank & Hurst 1996, Gemmell *et al.* 2004, cf. Keaney *et al.* 2019). This is expected to result

in local adaptation within populations through nuclear compensation for male function (Rand *et al.* 2004, Dowling *et al.* 2008, Ågren *et al.* 2019). Multiple studies find that males expressing foreign mitochondrial haplotypes incur fitness costs, without a parallel effect in females (Clancy *et al.* 2008, Camus *et al.* 2012, Camus *et al.* 2018, Nagarajan-Radha *et al.* 2019, reviewed in Vaught & Dowling 2018). Ideally, such an artefactual selection pressure is best avoided by backcrossing the cytoplasm of the source population into the CG females. However, the inextricable pairing of the compound X chromosome pair and the cytoplasm prevents us from introgressing the compound X into a desired cytoplasmic background. As a consequence, selected males are exposed to a foreign cytoplasmic background throughout the selection experiment. It is worth noting that Prasad *et al.* (2007) demonstrate that ML selection produces a male fitness benefit independent of cytoplasmic background and reversed sex chromosome inheritance, using test animals with control derived cytotypes and standard sex chromosome inheritance.

Abbott *et al.* (2013) provide insight into some of the artefacts included in the genetic background. In a study on the joint consequences of reversed sex chromosome inheritance, CG cytotypes and CG Y chromosomes on male fitness, they show that naïve males expressed through this system experience a fitness reduction compared to controls but adapt over the course of a selection experiment to improve beyond control fitness.

Given these selection artefacts, and the potential to adapt to them (through artificial arrest), we expect the selected males to adapt to the genetic background they are selected in and the CG females in the ML evolution experiment. We label this genetic background the “home court” (HC), in contrast to the “wild type” (WT) background that the controls remain in. Additionally, it is possible that these adaptations are even more specific – adapting to HC genetics and CG females in combination.

Here we report measures of competitive reproductive fitness (CRF), mating success, fecundity induction, sex ratio drive, and sperm offense. We study these traits in a fully factorial design using males in HC and WT genetic backgrounds, with both CG and non-CG females. We also study mate harm by selected and control males (in both genetic backgrounds) on CG females. Additionally, we study the fitness effects of the CG cytotype, Y chromosome and autosomes, each independent of the remaining artefacts in the genetic background.

We first address the hypothesis that these populations have little to no genetic variance due to long term domestication under stable conditions. If true, we predict no adaptive responses at all during the selection experiment, across all possible test conditions. If there are adaptive responses to the selection artefacts, we reject the hypothesis of no genetic variance, and identify artefacts that cause a selection response. Under these circumstances, we expect to see improved fitness in selected males due to local adaptation to arrested CG mates, the HC genetic background and the potential interaction - adaptation to CG females, in the HC background.

METHODS

ML Selection

Source populations, Selection treatment

The source populations and selection experiment protocols are described in detail in Thyagarajan *et al.*^a (chapter 2). Briefly, 3 replicate populations were derived from the control-old (CO) populations. From each, a male limited population was derived, through repeated crosses between “clone-generator” (CG) females and males from the ML population (fig A1a). Asexual propagation of the male limited genomes was avoided through a recombination system, that expressed a small percentage of the haploid genomes in females that carried two ML haploid genomes (fig A1b). The selection experiment was conducted for more than 80 generations.

Experimental males

The genetic mutations and aberrations used to accomplish male-limited evolution may cause reduced fitness, and therefore become targets for compensatory adaptation. We investigated these potential artefacts in combination and individually in males. All test males assayed carried target haplotypes, i.e., a haploid combination of all major chromosomes [I(X), II and III] from ML / MC populations. These were then paired with various “genetic backgrounds”, from the control or experimental treatments, as required. Where phenotypically marked control-derived competitors or females were required, we used lines backcrossed to have recessive eye colour markers (either “peach”, p^p , or recessive brown bw^l). Both competitor stocks are outbred and vigorous; here we refer to them collectively as “Cr”.

Males in “home court” and “wild type” conditions were assayed for competitive reproductive fitness (CRF), mating success, mating latency, copulation duration, fecundity induction, sex ratio drive, sperm offense, and mate harm. These were tested in combination with both Cr and CG females, in competition with Cr competitor males.

Test animals expressing a single artefact (explained below) were only assayed for CRF, to test for counter-adaptation by the ML males, or correlated adaptations in ML females. These were tested using competitions arenas with Cr males and females.

Home Court (HC) vs. Wild Type (WT) males

We expressed the target (ML and MC) haplotypes under ML selection conditions (HC) with genetic artefacts. To do this, we crossed MC and ML males to CG females. Red-eyed sons from these crosses carry a haploid genome from the target genotype, combined with a maternally inherited Y chromosome, cytoplasm, and translocated autosomes (fig B1a).

The ML and MC haplotypes were also tested without any of the genetic artefacts, resembling control animals (WT). The crosses conducted to produce these males are described in detail in Thyagarajan *et al.*^a (chapter 2) (fig B1b). Test animals for CRF assays combine the haploid target (ML or MC) genomes with control derived autosomes, Y chromosome, and cytoplasm. These are labelled ML & MC here but were referred to as ML_{SD} (single “dose” of target haplotype) and MC respectively in Thyagarajan *et al.*^a (chapter 2). WT flies in mating success and sperm offense trials (described below) were produced using a different breeding design (see fig B1c) (discussed in Thyagarajan *et al.*^a (chapter 2)).

Single artefact (Cyto / Auto / Y) males

To explore the impact of the potential genetic artefacts individually, we expressed target haploid genomes in combination with each CG derived genetic element individually,

drawing the remaining genetic background from control animals. We term these “CG-cyto”, “CG-auto” and “CG-Y” test animals respectively, based on the CG derived genetic artefact they carry.

About 60 generations into the selection experiment, we encountered a fortuitous mutant female derived from a spontaneous breakdown of the compound X chromosome pair, allowing us to tease apart the CG cytoplasm from the CX(DX) aberration. Using this mutant female, we backcrossed the CG cytoplasmic background into a stock homozygous for translocated autosomes (carrying a bw^D marker) and balancer X chromosomes (*FM7a*). Likewise, we also backcrossed the MC cytotype into the same stock, creating two cyto-lines labelled FMTW-CG (carrying the CG cytotype) and FMTW-C (carrying the MC cytotype). Female FMTW-CG 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 bw^D -marked translocated autosomes) were then crossed to MC males, to produce target male offspring carrying the CG cytoplasm, with control Y and autosomes (fig B2a).

To produce CG-Y males, we first crossed MC males to CG (bw^D) females. Sons from these crosses carried 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 bw^D 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 B2b).

To produce CG-auto males, we first backcrossed the CG males to MC females to create a line homozygous for CG translocated autosomes. We then used MC males to replace the CG derived Y chromosome while retaining the translocation. Males from this line were

crossed to females homozygous for ML and MC target haplotypes to produce target male offspring carrying the CG autosomes, with control cytoplasm and Y chromosome (fig B2c).

Experimental females

Single artefact (Cyto) females

We expressed ML and MC haploid genomes in female flies along with a single artefact at a time, to test for correlated responses to male counter-adaptations to the selection artefacts. While we found counter-adaptations to the cytotype and Y chromosome, we only study the influence of the cytotype on females, as Y chromosomes are male-limited by default. These flies carried a CG derived cytoplasm with MC derived X and autosomes (CG-cyto). The target females derived were tested for CRF.

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 CG (*bw^D*) females. Sons from this cross were crossed to the backcrossed females to produce target female offspring carrying the CG cytoplasm, with control autosomes (fig B3).

Assays

The designs used for CRF, mate choice and sperm competition assays are described in detail in Thyagarajan *et al.*^a (chapter 2). They are described briefly below, with notes on any deviations from the original design made to accommodate the CG females.

Competitive Reproductive Fitness (CRF) assays

CRF was measured as competitive reproductive success from mating arenas of 30 target animals combined with 30 Cr competitors of the same sex and 50 individuals of the opposite sex. Target animal output was measured by phenotyping the progeny using eye

colour. In assays using CG females, we assessed 10 such ‘contests’ 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. All male CRF assays were conducted between generations 64 - 72. CG-cyto female fitness was assayed in generation 83.

Mating success assays

Female mate choice was assayed (in generation 66) by combining a single female (CG or Cr) with a target male and Cr male. We identify the sire by phenotyping the progeny using eye colour. The resulting brood was also sexed and enumerated to test for fecundity induction and sex ratio drive effects. 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. These assays were conducted at ambient room temperature (slightly cooler than incubator), potentially resulting in slightly slower animals.

Sperm offense assays

Sperm offense (or P2) was assayed (in generation 74) by serially mating CG or Cr females first to males that carried the same markers and subsequently to target males. P2 was measured as the proportion of target male progeny. For each treatment (within each replicate pair of lines), we assess 30 such competition vials.

Mate harm assay

Mate harm was assayed (in generation 76) by measuring female mortality and productivity in response to being exposed to males. Following the crossing design, eggs of test animals are collected at densities of ~80 larvae per vial. Test males are collected on day 10-11. On day 12, 10 test males were combined in vials with 12 virgin CG females of the same age. Flies were held together for days 12-14, at which point the males were removed

from the females using light CO₂ 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 using pupal counts.

Statistical Analysis

Statistical analysis was conducted in R version 4.2.3 -- "Shortstop Beagle" (R Core Team 2023). Data was visually assessed for residual normality and using Bartlett's test for heterogeneity. Where generalised linear models were used, residual dispersion was tested using the DHARMA package (Hartig 2022).

Linear mixed models were employed to analyse CRF. For HC and WT males, selection, genetic background, female, and replicate population were used as fixed factors in the analysis to explain the proportion of offspring sired by the target individuals. To avoid pseudo-replication, contest was included as a random factor. Where significant interaction effects were identified, we employed planned contrasts between the levels of selection under each set of conditions with a corrected alpha value. For all the single artefact assays, selection and replicate were used as fixed factors, with contest used as a random factor. Where significant replicate differences were identified, we employed planned contrasts between the levels of selection in each replicate population pair with a corrected alpha value.

To study the mating success of target males in female mate choice trials, we used a generalized linear model with a binomial distribution. The proportion of mating successes for target animals was explained using selection, background, female, and replicate as fixed factors. Log(mating latency), mating duration, fecundity induction (offspring count) and sex drive (offspring sex ratio) were analysed using a Gaussian distribution, with selection, background, female, and replicate as fixed factors. Where significant replicate differences were identified, we ran models with the same fixed factors within each replicate level with a corrected alpha value.

For sperm offense, the proportion of offspring sired by the second male was analysed in two steps, as if in a Hurdle model. First, to create a data transformation that enabled the analysis, we subtract the proportion of offspring sire from 1. This inverted measure is then separated in two parts – values of zero (where all the offspring are sired by the target male) and non-zero values. The number of zeroes attributable to each treatment was analysed as a generalized linear model with a binomial distribution and using selection, background, female, and replicate as fixed factors. The non-zero proportion is analysed using a Gaussian distribution of errors, with selection, background, female, and replicate as fixed factors.

For mate harm data, we first modelled the decline in survivorship of females after the males were removed (day3-day7) using a linear model with selection, background and replicate as fixed factors. This was based on a visual observation the rate of female mortality slowed down after the removal of the males. This model showed no effect of selection, background or replicate on female mortality. Based on this, we made the decision to model the female mortality using data from day 0-day 2 (period of male exposure). Again, we use a linear model with selection, background and replicate as fixed factors. Likewise, we use a linear model with selection, background and replicate as fixed factors to analyse the productivity of the females mated to each treatment of males. Where significant interaction effects were identified, we employed planned contrasts between the levels of selection under each set of conditions with a corrected alpha value.

RESULTS

HC and WT animals

Competitive reproductive fitness

We find significant effects of selection ($p < 0.0001$) and background ($p < 0.0001$), along with significant interactions between selection and background ($p=0.0364$) and female and background ($p < 0.0001$) on CRF (table B1, fig 3.1c, d). We found no effect of replicate, or any interaction with replicate. The main model does violate Bartlett's test for variance homogeneity ($p < 0.0001$). Analysing the effect of selection within each combination of the levels of female and background, we find the following (see table B1a, fig 3.1a, b). In HC conditions with CG females, we find a significant effect of selection ($p < 0.0001$), and no effect of replicate or interaction with replicate. ML males displayed a CRF of 0.224 ± 0.035 (mean $\pm 1.96 \times \text{se}$), compared to MC males at 0.110 ± 0.025 . In HC conditions with Cr females, we find a significant effect of selection ($p < 0.0001$), and no effect of replicate or interaction with replicate. ML males displayed a CRF of 0.295 ± 0.015 compared to MC males at 0.178 ± 0.010 . In WT conditions with CG females, we find no effect of selection, replicate or interaction. Selection ($p = 0.0189$) approached the adjusted $\alpha = 0.0127$ threshold for 4 comparisons. ML males displayed a CRF of 0.468 ± 0.038 , compared to MC males at 0.372 ± 0.033 . In WT conditions with Cr females, we find no effect of selection, replicate or interaction. ML males displayed a CRF of 0.341 ± 0.018 , and MC males 0.322 ± 0.017 .

Mate choice

We find significant effects of selection ($p < 0.001$), female ($p = 0.014$), and replicate ($p = 0.001$) on the success of target males mating success (table B2a). We find no effect of background or interaction effects. Within replicate 1, we find a significant effect of selection ($p = 0.016$) and no other effects. ML males across both backgrounds and females displayed a success rate of 63.93%, compared to MC males at 51.58%. In replicate 3, we find no

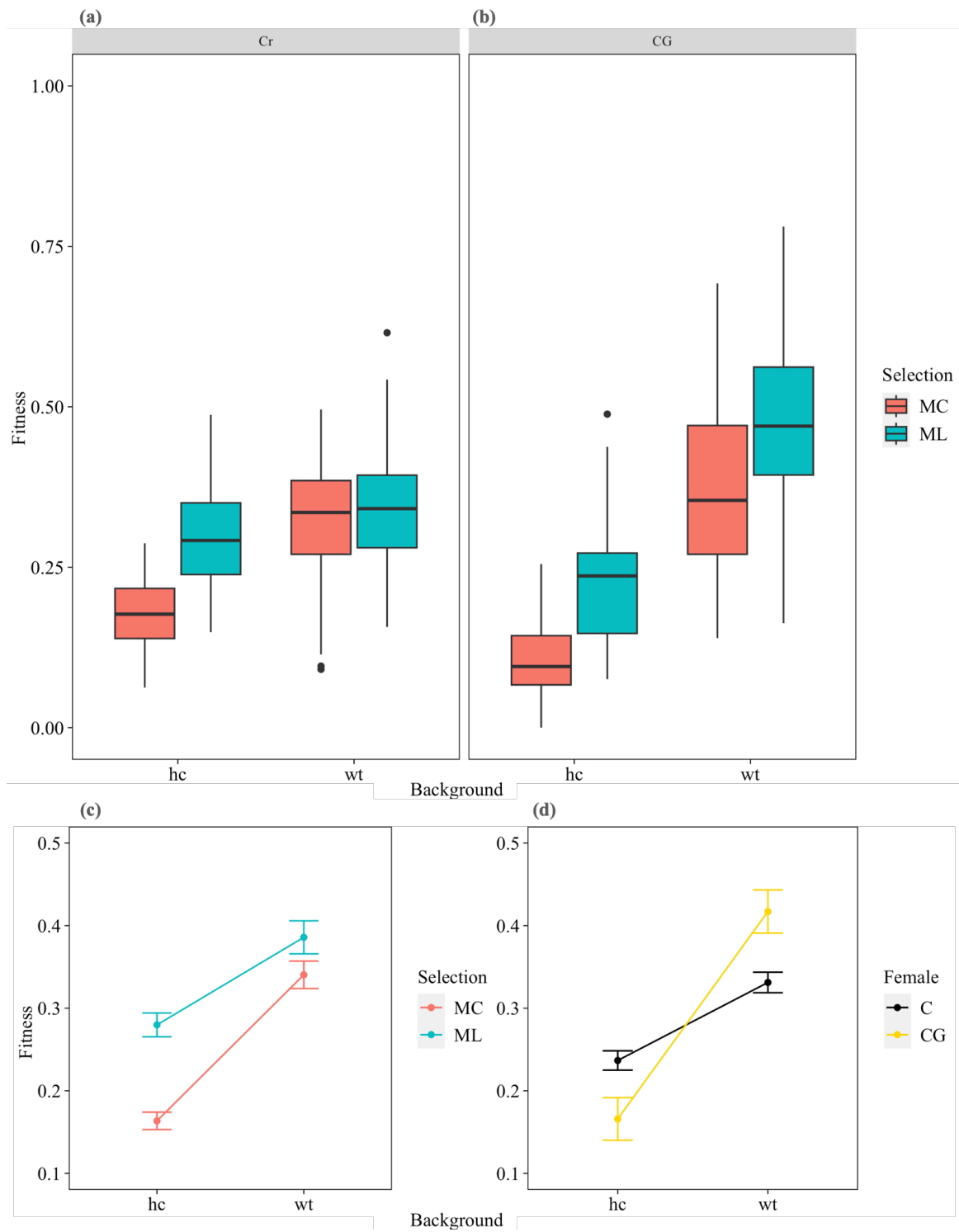


Figure 3.1. CRF of target males in hc (home court - ML like) & wt (wild type - MC like) genetic backgrounds, tested against (a) recessively marked control females and (b) clone generator females used in the ML selection experiment. Panels (c) & (d) highlight the interaction between (c) selection and genetic background and (d) female and genetic background.

significant effects at all, but selection ($p = 0.032$) approached the adjusted $\alpha = 0.0170$ threshold for 3 comparisons. Here, ML males displayed a success rate of 60.10%, compared to MC males at 48.62%. In replicate 5, we find no significant effects. Here, ML males displayed a success rate of 48.40%, compared to MC males at 40.98% (table B2b, fig 3.2a).

Our analysis of mating latency reveals a significant effect of selection ($p=0.028$) (table B2c). We find no effect of female, background, replicate or interactions on latency. ML males across all conditions displayed a latency of 14.05 +/- 2.08 minutes, compared to MC males at 17.17 +/- 2.89 minutes (fig 3.3a).

Copulation duration on the other hand reveals significant effects of selection ($p = 0.017$), background ($p < 0.001$), female ($p < 0.001$) and replicate ($p = 0.020$), but no interaction effects (table B2d). Analysed within each replicate with an adjusted alpha threshold though, we find no effect of selection (or any interaction with selection) in any of the replicates (table B2e). Overall, ML males mate for a duration of 19.33 +/- 0.53 mins, compared to MC males at 20.33 +/- 0.66 mins (fig 3.3b). In replicate 1, the durations are 20.07 +/- 0.87 (ML) and 20.57 +/- 0.95 mins (MC) respectively. In 3, it is 19.38 +/- 0.82 and 20.65 +/- 1.27 mins and in 5, it is 18.27 +/- 1.04 and 19.68 +/- 1.27 mins.

Fecundity induction, sex ratio drivers

Our analysis of fecundity induction reveals significant effects of background ($p < 0.0001$), female ($p < 0.0001$), replicate ($p = 0.013$), and an interaction between female and background ($p < 0.0001$) (table B2f, g). Across all possible conditions however, we find no effect of selection, nor any interaction with selection, with the mates of ML males producing 27.80 +/- 2.20 offspring compared to the mates of MC males at 27.18 +/- 2.46 (fig 3.3c).

We find no significant differences across any of the factors studied or their interactions, in our analysis of sex ratio drive (table B2h). ML males produce broods with a sex ratio (male/total) of 0.505 +/- 0.011, compared to MC males at 0.494 +/- 0.011 (fig3.3d).

Sperm offense

Across both steps of our sequential model to analyse sperm offense, we find no effect of selection or significant interaction with selection on P2 (tables B3a, b). In both steps, we find significant effects of background, female and an interaction between female and background. Overall, MLs display a P2 of 0.848 +/- 0.0169 compared to and MC 0.825 +/- 0.0175 (fig 3.2b).

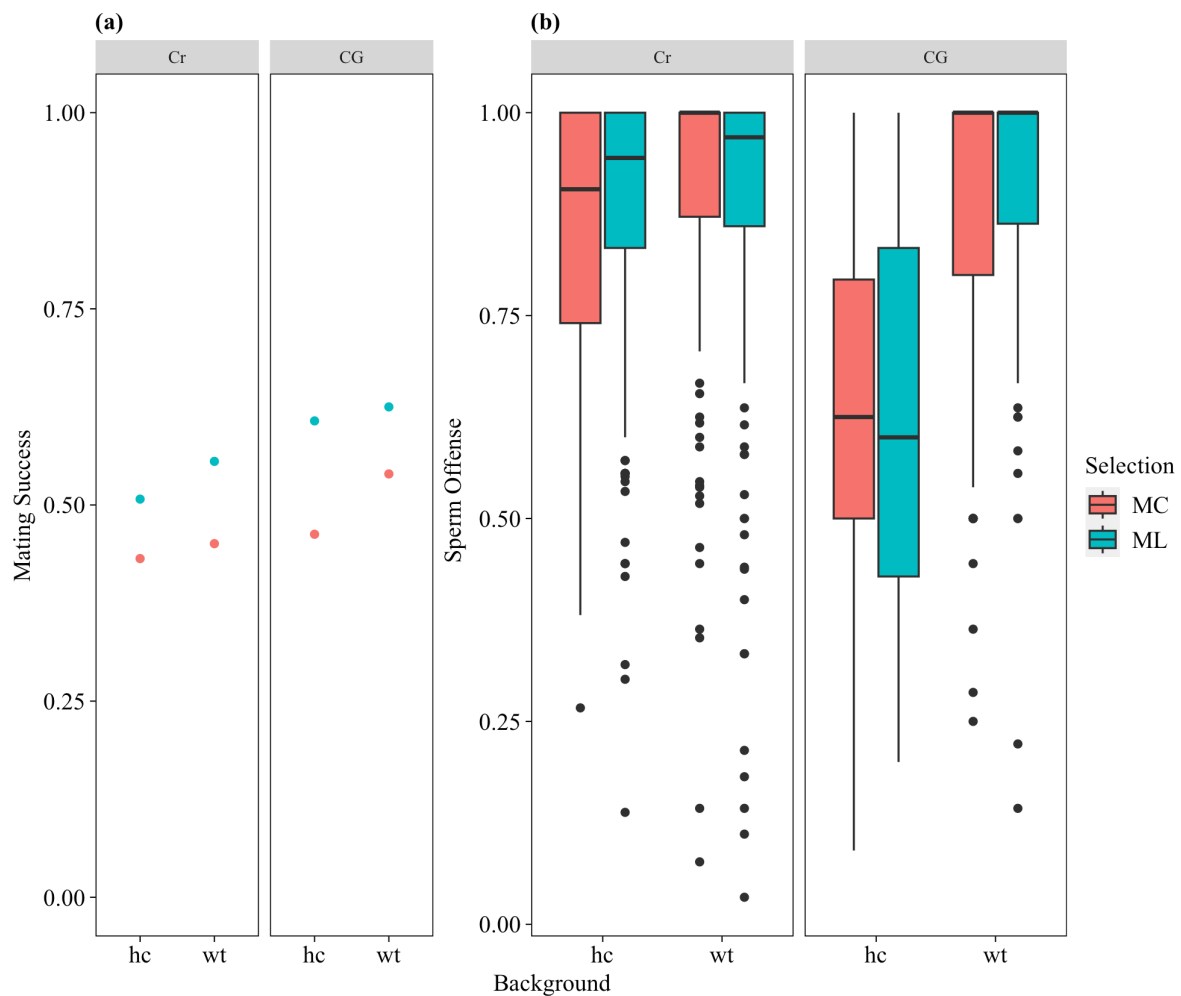


Figure 3.2. (a) The proportion of mating success of target males and (b) the proportion of offspring sired by target males as second mate (P2 - sperm offense). Target males from each genetic background (hc & wt) are tested against both kinds of females (recessive competitors - Cr, and clone generators - CG).

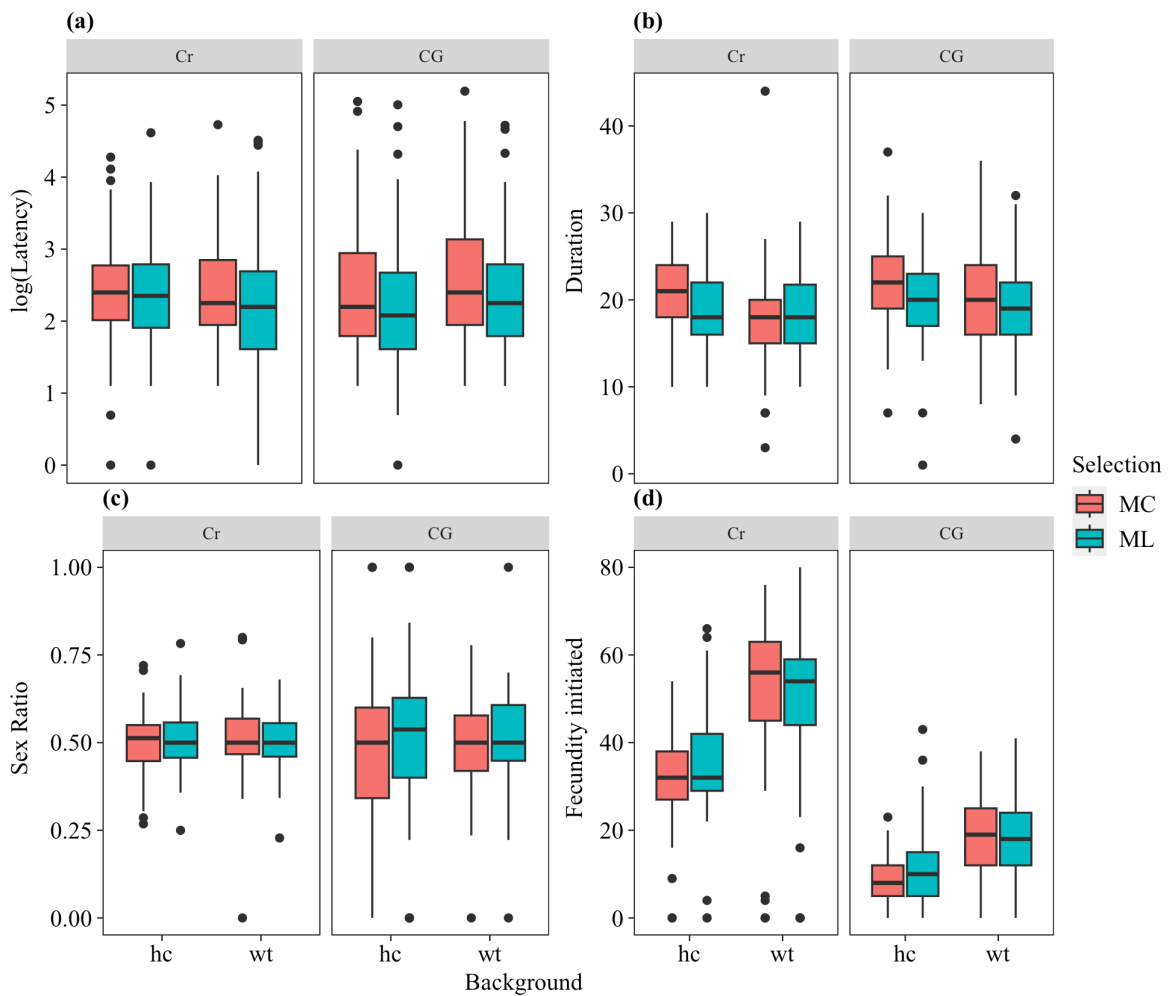


Figure 3.3. Measures of (a) mating latency, (b) mating duration, (c) brood sex ratios and (d) fecundity induction for target males from each genetic background (hc & wt), tested against both kinds of females (recessive competitors - Cr, and clone generators - CG).

Mate harm

Both selection ($p < 0.0001$) and background ($p < 0.0001$) had a significant effect on female mortality during male exposure. We found no effect of replicate, or any interaction effect (table B4). Under HC conditions, ML males induced an average rate of mortality of 2.40 ± 0.365 , compared to MC males at 3.20 ± 0.529 . Under WT conditions, ML males induced an average rate of mortality of 1.35 ± 0.326 , compared to MC males at 2.37 ± 0.470 (fig 3.4a).

Our analysis of productivity revealed significant effects of selection ($p < 0.0001$) and background ($p < 0.0001$), and a significant interaction between selection and background ($p < 0.0001$). We found no effect of replicate, or any interaction with replicate (table B4). Analysing productivity under each background independently, we found no effects of selection, replicate or their interaction under HC background, but a significant effect of selection ($p < 0.0001$) under WT conditions.

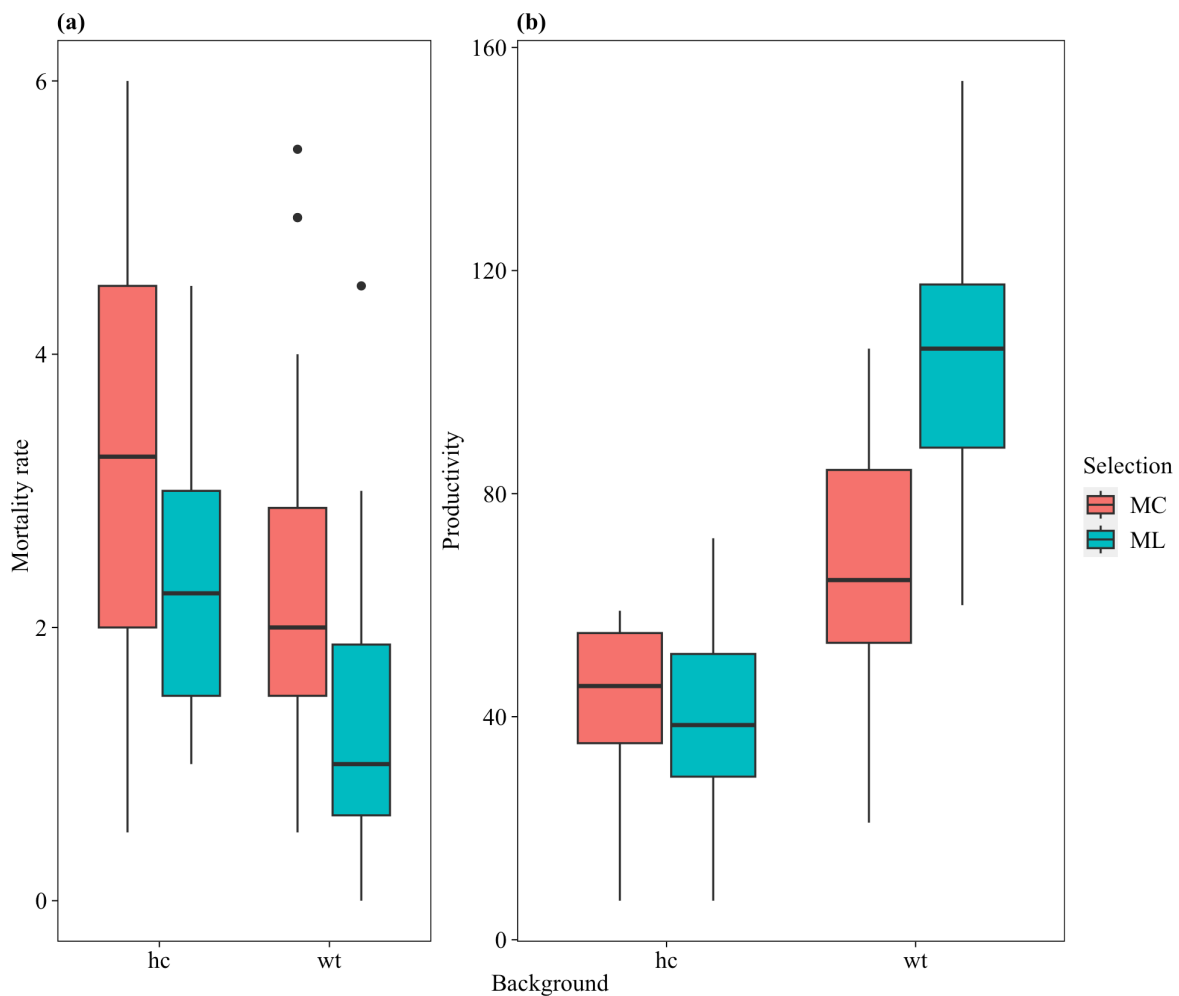


Figure 3.4. Clone generator (CG) female (a) mortality per diem during male exposure and (b) pupal productivity upon exposure to target males from both home court (hc) and wild type (wt) genetic backgrounds.

Neither replicate nor interaction between selection and replicate were significant under WT conditions. In the HC background, ML and MC males induced productivities of 40.0 ± 5.36

and 43.5 +/- 4.76 respectively. In the WT background, ML and MC males induced productivities of 104.0 +/- 7.54 and 68.0 +/- 8.33 respectively (fig 3.4b).

Single artefact animals - CRF

CG Autosomes

We find no effect of selection, replicate, or interaction between selection and replicate on CRF of males with the CG autosomes (table B5a). ML males displayed an overall CRF of 0.447 +/- 0.019, compared to MC males at 0.405 +/- 0.016 (fig 3.5c).

CG cytoplasm

We find a significant effect of selection ($p = 0.02$) on CRF of males with the CG cytotypotype (table B5b). There was no effect of replicate, or interaction between selection and replicate. ML males displayed an overall CRF of 0.360 +/- 0.019, compared to MC males at 0.304 +/- 0.016 (fig 3.5b).

We find a significant effect of replicate ($p < 0.0001$) and interaction between selection and replicate ($p < 0.001$) on CRF of females with the CG cytotypotype (table B5e). Selection was found to be not significant ($p = 0.98$). When analysed by replicate, we find that selection has a significant effect on CRF at the adjusted $\alpha = 0.0170$ threshold in replicates 5 ($p = 0.0004$), but not in 1 ($p = 0.93$) and 3 ($p = 0.85$) (table B5f). In replicate 1, MLs and MCs displayed CRFs of 0.488 +/- 0.017 and 0.488 +/- 0.016 respectively. In replicate 3, it was 0.494 +/- 0.017 and 0.497 +/- 0.014. In replicate 5, it was 0.516 +/- 0.014 and 0.569 +/- 0.015 (fig 3.5d).

CG Y

We find a significant effect of selection ($p < 0.0001$), replicate ($p < 0.0001$) and interaction between selection and replicate ($p < 0.01$) on CRF of males with the CG Y chromosome (table B5c). When analysed by replicate, we find that selection has a significant effect on CRF at the adjusted $\alpha = 0.0170$ threshold in replicates 1 ($p = 0.0011$) and 3 ($p <$

0.001), but not in 5 ($p = 0.7065$) (table B5d). In replicate 1, MLs and MCs displayed CRFs of 0.431 ± 0.029 and 0.312 ± 0.017 respectively. In replicate 3, it was 0.340 ± 0.026 and 0.198 ± 0.020 . In replicate 5, it was 0.357 ± 0.029 and 0.342 ± 0.033 . (fig 3.5a)

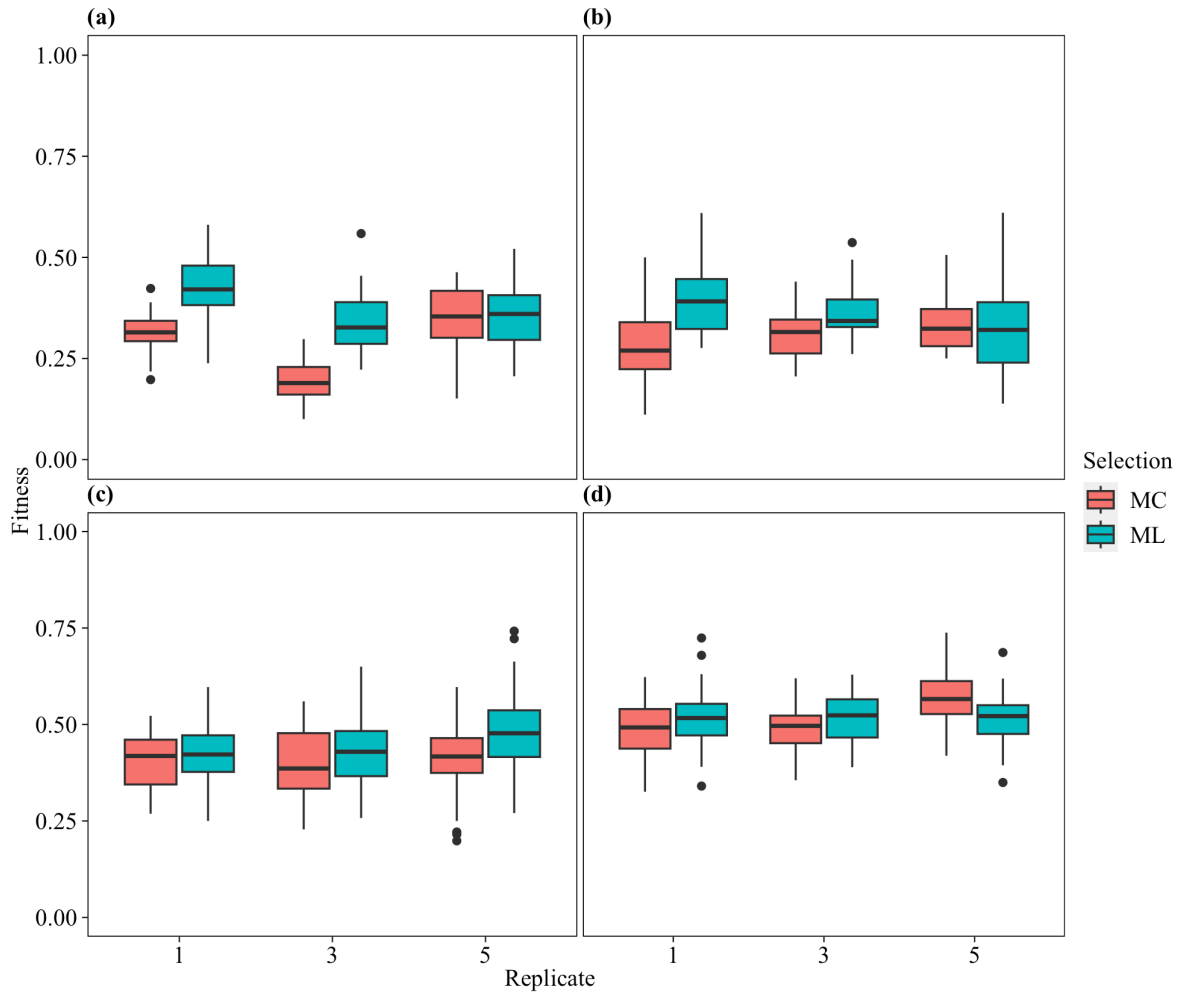


Figure 3.5. Competitive reproductive fitness (CRF) of target males with a completely control genetic background except for (a) CG-autosomes, (b) CG-cytoplasm, (c) CG-Y chromosome; and CRF of target females with a completely control genetic background except for (d) CG-cytoplasm.

DISCUSSION

We recently reported that a new application of the ML sex-limited selection protocol provided little evidence for the resolution of intralocus sexual conflict in three newly investigated populations of *D. melanogaster*. This result left us wondering if long-term domestication had depleted genetic variation in the base populations used to initiate the experiment. Here we present evidence for a marked response to selection in these populations that is dependent upon both the genetic background of the test males and the females they interact with: Male-limited evolved males (ML) were twice as fit as their MC controls (+103.6%) when tested with all of the features of their selection protocol, including competing for the special clone-generator (CG) females they had potentially become specialized on. With control females, ML-evolved males genetically in their “home court” were about 66% fitter than controls. While the selected males show no significant fitness advantage in the “wild type” genetic background, there is a nearly significant trend of improved fitness with CG females (25.8% improvement). These results suggest extensive compensatory adaptation to the cytogenetic methods employed in ML-selection and to the clone-generator females that the males interacted with. Strong selection from sources unrelated to the removal of balancing SA selection likely obscures our ability to detect IaSC. As suggested by Rice (1996), the CG females represent a “non-responding target” for ML males, with coevolution between the sexes disrupted in favour of male specialization and exploitation. Here we break down these general features of the selection response, showing the roles of individual selection artefacts and considerable variation across replicate populations.

The fitness increase in selected males is primarily explained by increased mating success in competition (although not consistently across replicates), with no changes between selected and control lines in fecundity induced, brood sex ratio or sperm offense across

genetic backgrounds and female identity. When mating with the frail CG females, selected males also cause less mate harm than controls in both genetic backgrounds, and indirectly improve their own reproductive success.

We find that the fitness of selected lines improves in the presence of the CG cytotype and the CG Y chromosome (although not consistently across replicates), but not in the presence of the CG translocated autosomes. Adaptation to the CG cytotype in the males results in correlated benefits to the females (although not consistently across replicates), suggesting that these adaptations are not sexually antagonistic or male limited.

Our findings reject the hypothesis that the base populations used in the selection experiment have no genetic variance. Additionally, we find that selected animals show trends of improvement even in the WT background when tested with CG females. This suggests that the absence of a fitness phenotype in Thyagarajan *et al.*_a (chapter 2) is not due to the ML haplotype being completely recessive to the MC haplotype. We address this in greater detail through phenotypic traits in Thyagarajan *et al.*_c (in prep).

The increased fitness of the selected males against CG females is consistent with findings from previous male limited selection experiments (Rice 1996, Prasad *et al.* 2007). This is in alignment with the expectation of local adaptation to a novel environment of sexual selection for males, likely accelerated by the arrest of female co-evolution. To study how these males adapt to the females used in the breeding design, we look for interaction effects with tester female identity across the traits assessed. We find that this adaptation is not mediated through fecundity induction, sex ratio drive or sperm offense, as we see no differences between selected and control lines in any of these traits across both genetic backgrounds. Curiously, while we find differences in mating success between selected and control animals, our analysis shows no interaction between selection and tester female identity in this model. However, as noted in Thyagarajan *et al.*_a (chapter 2), the breeding

design we use to produce WT test animals for mate choice and sperm competition assays differs slightly from that used in the CRF assay. This design results in target males that include a CG derived Y chromosome. We argue that the presence of this Y chromosome results in mating success advantage for selected males even in the WT background with Cr females (where it is not expected based on CRF data), obscuring our ability to identify an interaction between selection and female identity. This CG Y based fitness benefit is corroborated by the increased fitness of selected animals in the CG Y fitness assay, and the increased mating success of WT animals described in Thyagarajan *et al.*^a (chapter 2). Based on these lines of evidence, we speculate that the adaptation to these females occurs through increased mating success in selected lines, rather than sperm offense or fecundity induced. This aligns with the findings of Jiang *et al.* (2011), who find no sperm competitive advantage for selected males with CG tester females, but is in contrast with Rice (1996), where sperm offense and defense both showed marked improvements.

Further, we find that the selected males show lower levels of mate harm than controls when combined with CG females, regardless of genetic background. This too is in contrast with the findings of Rice (1996), where selected males were shown to cause greater levels of harm of the CG females they mated with. In the WT background, this reduced harm also allows for improved female productivity, indirectly improving the fitness of the selected males. This result is especially fascinating, as evolved reductions in mate harm have only been identified in response to reduced intensity of IeSC (as with monogamy selection - Holland & Rice 1998).

As predicted, our assays on males carrying a single artefact show that both the CG Y chromosomes and CG cytotype have significant effects on male fitness, with selected males showing higher fitness than controls in both situations. We interpret this as selected haplotypes undergoing compensatory adaptation to selection pressure from the “mother’s

curse” (Gemmell *et al.* 2004) in the presence of a foreign cytoplasm, and a Y chromosome that has likely undergone selection to suit both female and male function.

Previously, we showed that female fitness of selected haplotypes declined under WT conditions (Thyagarajan *et al.*_a (chapter 2)). Here, we ask if compensatory adaptation in the males takes advantage of sex-limited selection to improve males through routes that are indifferent (sex-limited) or antagonistic to female fitness. While we identify male adaptation to the CG cytotype and Y chromosome, we only test female fitness in the presence of the CG cytotype. When tested, we found a recovery of fitness in the selected haplotypes relative to controls, suggesting that the nuclear response to mother’s curse in selected haplotypes produces sexually concordant benefits.

We note that these compensatory effects are not consistent across replicate. While selected males in replicate 5 show little to no improvement over controls with both the CG cytoplasm and CG Y chromosome, we find clear improvements in both cases for replicates 1 and 3. Likewise, while females with selected haplotypes carrying CG cytotype show fitness recovery in replicates 1 and 3, such a recovery is not observed in replicate 5. Further, while we find no significant effect of selection overall when assessing the effect of the CG autosomes on fitness, we observe that the selected animals in replicate 5 show a trend of improved fitness relative to their paired controls under these conditions. In Thyagarajan *et al.*_a (chapter 2), we report the CRF of animals that express only the autosomes of a target haplotype, with a control X chromosome (referred to as MLSD(a)). Here, we found that the while MLSD(a) animals in replicates 1 and 3 show a trend of reduced fitness relative to the controls and MLSD (ML haplotype - single dose) animals, the MLSD(a) males of replicate 5 display the same fitness as MLSD animals, suggesting a role for autosomal adaptation. Taken together, the 3 replicate populations follow different routes in adapting to the myriad selection pressures introduced by this experiment.

The compensatory adaptation displayed in the selection experiment raises concerns about previous experimental work conducted using CG flies. CG flies have been 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 *et al.* 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; Abbott *et al.* 2020) and male-limited selection experiments (Rice 1996; Prasad *et al.* 2007). It is important to note that most of these experiments (all but McKean *et al.* 2008, Mallet *et al.* 2011 & Tennant *et al.* 2014) work with target haplotypes derived from the LH_M stock population, which might not undergo artefactual selection pressures described herein. In different iterations, authors have taken various level of precautions against possible artefacts, ranging from Rice (1998_a) 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 CG derived cytotypes and Y chromosomes, frequently inherited directly from the dam. In this work we find that both can introduce fitness effects, differentially affecting selected and control animals in selection experiments, potentially distorting trait variance in hemiclinal analyses.

Here, we propose improved designs that could allow us to successfully interpret results from such experiments. In Thyagarajan *et al.*_a (chapter 2), we describe a method to conduct a hemiclinal analysis that controls for all artefacts except the CG-derived Y chromosome. We propose that this method, combined with backcrossing of a control derived Y chromosome into CG lines a generation before assay is best suited to limiting artefactual noise in estimating trait variances and intersex genetic correlations. For male limited selection experiments, we suggest the use of a control population backcrossed to carry the CG cytotype, combined with regular backcrossing of a control Y chromosome into the CG

populations. Further, female coevolution can be released from “arrest” by simply collecting the CG daughters of the ML males (through careful examination for necessary genetic markers) to serve as dams for the next generation of ML selection. However, even with a more demanding design, we cannot account for the effects of reversed sex chromosome inheritance, or any potential interaction effects between the constructs in the genetic background. In fact, such a selection design would offer an opportunity to isolate reversed sex chromosome inheritance and examine evolved changes in epigenetic effects – potentially illuminating the contribution of existing epigenetic effects to fitness.

CHAPTER 4

Evolution of reproductive isolation in a long-term evolution experiment with *Drosophila melanogaster*: 30 years of divergent life-history selection.

ABSTRACT

We ask if three decades (and over 1,500 generations) of divergent life history selection on age at reproduction has resulted in the evolution of reproductive isolation (RI) between laboratory populations of *Drosophila melanogaster*. We tested for premating, postmating-prezygotic and postzygotic reproductive isolation between 3 replicate population pairs. Large, evolved differences in body size between selection treatments suggested the potential for prezygotic barriers driven by sexual selection or physical incompatibilities between the sexes. Although a simple prediction would be preference for larger size, creating directional isolation, our results from individual mate choice trials indicate that populations from both selection treatments show a marked bias towards homotypic mate choice; indicative of prezygotic RI driven by sexual selection or sexual conflict. Hybridization between the focal populations resulted in the production of viable adult flies with intermediate size and developmental traits. We observed a suggestive but statistically non-significant trend of fitness decline in the F2 generation of hybrids, but no significant evidence suggesting the evolution of postmating-prezygotic or postzygotic RI. Our findings are in accord with extant literature that posits that premating RI evolves before postmating forms of RI.

INTRODUCTION

Understanding the mechanisms underlying the evolution of reproductive isolation (RI) and the timing of their origination is central to the study of speciation. With the fossil record yielding limited information about this process on microevolutionary timescales, there has been a focus on extant incipient species, either where populations naturally intermingle in contact zones or through experimental crosses. Such populations, part way through the process of RI, can help to illuminate the nature of divergence, as indicated by pre-mating isolation or hybrid inferiority due, for example, to antagonistic epistasis between incompatible alleles (Dobzhansky 1937, Muller 1942) and hence offer a snapshot of speciation in action. But what selective agents or historical contingencies have driven the divergence may or may not be apparent, particularly as replication and controls may be absent or limited, and partial isolation and changes in the environment can lead to reversals in the development of RI (Lackey & Boughman 2017), complicating the inference of sequence in the evolution of isolating mechanisms.

Here we focus on early stages of ecological speciation. Ecological speciation is the process through which reproductive isolation evolves as a consequence of local adaptation with limited gene flow. Divergent selection pressures can contribute to this process by acting directly, or through pleiotropy or tight linkage with genes that are directly responsible for reproductive isolation (Rice & Hostert 1993, Rundle & Nosil 2005). Divergent evolved responses between ecotypes may result in (1) changes in morphology, mating behaviours or physiological characters that hinder mating attempts mechanically or via mate choice (pre-mating reproductive isolation); (2) changes in gametes/reproductive machinery that inhibit fertilization (post-mating-prezygotic reproductive isolation); and/or (3) genetic incompatibilities that reduce survival or reproductive success of hybrid individuals (postzygotic reproductive isolation).

In addition to divergent natural selection in allopatric populations, divergent intersexual coevolution via sexual selection has long been recognized to have a potentially significant role in the evolution of reproductive isolation (Lande 1981, Panhuis *et al.* 2001, Ritchie 2007, Maan & Seehausen 2011, Singh & Singh 2014). Likewise, interlocus sexual conflict – where traits that increase the fitness of one sex directly cost the other – can result in rapid coevolution (an “evolutionary chase” or “arms race”) between male and female reproductive traits (Rice & Holland 1997, Parker & Partridge 1998, Rice 1998) and potentially accelerate the process of speciation (Ritchie 2007, Gavrilets 2014, Syed *et al.* 2017); the evolution of female resistance to reduce the direct costs of mating is predicted to contribute to assortative mate choice (Gavrilets *et al.* 2001).

While natural systems such as hybrid zones have advanced our understanding of the evolution of RI immensely, researchers are frequently limited to indirect inferences, such as using estimates of gene flow and clines to infer reproductive isolation. Beyond knowledge of the evolutionary history, these systems limit the number RI barriers that can be assessed, and generally lack replication at the population-pair level. Notable exceptions include stickleback fishes, where repeated invasion of freshwater from marine ancestors, and subsequent differentiation into limnetic-benthic and lake-stream ecotypes provide extensive replication (Lackey & Boughman 2017); and storm-petrels, where repeated separation of populations by breeding time (allochrony) provides replicated evidence of reproductive isolation in sympatry (Wallace *et al.* 2017). Experimental evolution can be a valuable tool for more direct investigation, allowing us to probe potential drivers of speciation between populations with well understood evolutionary histories. Experimental studies in speciation have attempted to test if (1) non-ecological drivers like drift, (2) negative selection against hybridization between ecotypes, (3) divergent selection on populations or (4) sexual conflict produced reproductive isolation (reviewed in Rice and Hostert 1993, Fry 2009, White *et al.* 2020). An

obvious limitation to experimental evolutionary studies of speciation is time: research projects rarely exceed granting cycles and few organisms lend themselves to experimental evolution. It is little surprise then that *Drosophila* is far and away the genus most investigated in the laboratory.

Early divergent selection studies produced seemingly equivocal results with respect to creating reproductive isolation. A closer examination suggested that divergent selection was highly effective, given a pre-existent tendency to mate assortatively on the basis of the trait selected upon (Rice and Hostert 1993, Fry 2009). Divergent selection upon behavioural traits resulted in the correlated evolution of reproductive isolation (del Solar 1966, Soans *et al.* 1974, Hurd & Eisenberg 1975, Lofdahl *et al.* 1992) while divergent selection on other traits like bristle number (Barker & Cummins 1969, Santibañez & Waddington 1958) produced negative results. More recently, experimental evolution studies have focused on selection regimes that do not directly involve characters that are known to influence intra-population assortment. For example, a number of studies have investigated the importance of local adaptation to distinct nutrition sources for mating assortment. Divergent selection regimes with different nutritional environments result in premating isolation through (1) the evolution of signalling traits and mating preferences in *Drosophila serrata* (Rundle *et al.* 2005), (2) the evolution of symbiotic microbiota in inbred strains of *D. melanogaster* (Najarro *et al.* 2015, Sharon *et al.* 2010), (3) competitive ability differences between controls and selected individuals in *D. melanogaster* (Belkina *et al.* 2018), (4) positive assortative mating preferences in *D. melanogaster* (albeit unstable) (Nash *et al.* 2019) and in *Saccharomyces cerevisiae* (Mahilkar *et al.* 2023); and (5) postzygotic reproductive isolation through genetic incompatibilities in *Saccharomyces cerevisiae* (Dettman *et al.* 2007) and *Neurospora* (Dettman *et al.* 2008).

Life history traits are less frequently studied in the context of reproductive isolation, but have also received some attention – predominantly in studies of allochronic speciation. Powell *et al.* (2020) note how adaptation to differences in seasonal timing has been shown to contribute to reproductive isolation in a wide variety of organisms including plants (Savolainen *et al.* 2006, Lowry *et al.* 2008), insects (Tauber *et al.* 1977, Wood & Guttman 1982, Horner *et al.* 1999, Eubanks *et al.* 2003, Abbot & Withgott 2004, Forbes *et al.* 2009, Ording *et al.* 2009, Wadsworth *et al.* 2013, Hood *et al.* 2015) and vertebrates (Friesen *et al.* 2007). Speciation through other life-history traits and correlated changes in intersexual coevolution has received less attention, due to the myriad challenges associated with studying these systems in nature. Divergent selection on pre-adult development time has been shown to produce pre-mating isolation through (1) disturbances in circadian rhythm resulting in changes in phases of mating in the melon fly, *Bactrocera cucurbitae* (Miyatake & Shimizu 1999), and (2) evolved body size differences, and correlated levels of sexual conflict, in *Drosophila melanogaster* (Ghosh & Joshi 2012). Likewise, divergent selection on body size results in premating isolation, through mechanical incompatibilities during attempted copulations between *Columbicola* feather mites (Villa *et al.* 2019).

Age-at-reproduction has been extensively manipulated using experimental evolution in *Drosophila* to investigate correlated changes in longevity, development time, body size and a slew of associated traits (Prasad & Joshi 2003). In this study, we use an exceptionally long term evolution experiment (LTEE) with population pairs separated by over 1,500 generations and three decades under divergent selection in allopatry. The two *D. melanogaster* life-history selection regimes used in this study are selected to reproduce late in life (4 weeks from egg; CO) and extremely early in life (9 days from egg; Accelerated CO / ACO). The ACO populations have evolved small body size, rapid development time, and reduced lifespan (Chippindale *et al.* 1997a, Chippindale *et al.* 2004a). On the other hand, the CO flies

are big, develop slowly and likely have an increased ability to resist copulations as females and to inflict harm as males (Chippindale *et al.* 1997b, Chippindale *et al.* 2004b, Mital *et al.* 2022, Verma *et al.* 2022).

In the speciation literature, body size is identified as a crucial trait that can drive the evolution of reproductive isolation (Servedio *et al.* 2011). In *D. melanogaster*, body size is strongly correlated with juvenile development time (Zwaan *et al.* 1995a, Nunney 1996, Chippindale *et al.* 1997a, Prasad *et al.* 2000, Prasad & Joshi 2003) and life-history selection on development has been shown to result in the evolution of body size (Chippindale *et al.* 1997a; Prasad *et al.* 2000). An important sexually selected trait for both sexes, large females tend to be more fecund (Stearns 1992, Roff 2002) and attract higher courtship effort from males (Byrne & Rice 2006); while large males have increased competitive abilities (Partridge & Farquhar 1983, Markow & Ricker 1992), and secure more frequent copulations as a consequence of female preference (Markow 1986; Partridge *et al.* 1987, but see Prasad *et al.* 2007). Given the evolved size differences between the ACOs and COs, a simple prediction is that individuals of both population-types display a mating preference for individuals from the larger CO populations, producing conditions of partial, directional, reproductive isolation driven by sexual selection on the basis of body size, along the lines observed by Ghosh and Joshi (2012).

In *D. melanogaster*, divergent reproductive strategies between the sexes have long been recognized (Bateman 1948) potentially resulting in interlocus sexual conflict. Frequent matings increase male fitness but can decrease female fitness as a result of male harassment and the harmful effects of genital wounding and seminal fluid (Chapman *et al.* 1995, Bonduriansky *et al.* 2008, Travers *et al.* 2015). The harm experienced by females as a result of mating, measured by lifespan and egg production rate, is correlated with male body size (Pitnick & García-González 2002, Friberg & Arnqvist 2003). In the context of our two

selection treatments, we predict that male harm and female resistance traits will be stronger in the CO populations compared to the ACO populations, due to the difference in body size between the large COs and smaller ACOs. In experimental hybridization between CO and ACO lines, this difference could drive premating isolating barriers to evolve in one direction, with CO females resistant to mating with the smaller ACO males, and ACO females vulnerable to unwanted mating with CO males. Indeed, working with a similar system, Ghosh and Joshi (2012) found such one-directional isolation, driven by large females resisting mating attempts by small males, and a preference for (or lack of resistance to) large males in females of both treatments. On the other hand, especially with the large number of generations in allopatry, mating signals and preferences may have diverged between populations in our selection experiment creating the possibility for positive assortment in mate choice experiments.

We investigated mate choice through individual female, male, and group mating choice assays in the ACO/CO complex of selected lines. We further looked for potential gametic incompatibilities (postcopulatory prezygotic isolation) by comparing egg hatchability in hybrid matings to the parental populations. Finally we looked for postzygotic effects (hybrid breakdown or vigour) in developmental traits, body size, and adult fitness.

METHODS

Experimental populations

Our experiments employed the accelerated development (ACO) (Chippindale *et al.* 1997a) and control-old (CO) (Rose *et al.* 1992) populations. All populations are maintained under standard laboratory conditions: moderate density (larval density of 80 - 120 larvae / vial), 25°C, 12:12 light:dark cycle on banana/agar/killed-yeast medium, and with a census population size of approximately 2,000 individuals per generation.

The five CO populations (CO₁-CO₅) were derived from like-numbered O populations in 1989 (Rose *et al.* 1992). Each CO population is maintained on a 28-day discrete life cycle, with flies placed into cages at a density of ~1000 flies/cage. On day 26, yeast paste is added to the medium to stimulate egg-laying, and on day 28/day 0, eggs are collected at 80-120 eggs per vial. In 1991, one A population (ACO₁-ACO₅) was derived from each of the five CO populations and initially subjected to direct selection for accelerated development. After generation 175, these accelerated populations were maintained on a discrete life cycle of 9 days.

Experimental populations were derived from three of the five pairs of ACO and CO populations (ACO₁, ACO₃, ACO₅ and CO₁, CO₃, CO₅). To remove environmental effects and synchronise experiments, all populations were maintained over a control 14 day life cycle for two generations prior to any assay. Prior to experiment, life cycles were staggered in light of evolved development time differences (ACO << CO) with a 48h difference in egg collection time to synchronise eclosion time of adults. Each assay was repeated for three replicate population pairs: CO₁-ACO₁, CO₃-ACO₃, and CO₅-ACO₅.

Female mate choice assay

To test for the existence of premating reproductive isolation between populations as mediated by female preference, we evaluated the mate choice of ACO and CO females. Flies were sorted by sex and collected as virgins within 6 hours of eclosion under light CO₂ anaesthesia. A food dye technique was used to differentiate between males from each regime (developed from Verspoor *et al.* 2015). Dye techniques were calibrated based on tests of mortality, and the volume of dye used did not result in detectable harmful side-effects, measured through changes in lifespan. Vials were dyed three days prior to the assay using 6 drops of red or blue food dye. One day prior to the experiment, male flies were added to coloured vials at a density of 10 flies/vial and left to ingest the dye for 24 hours. Females were acclimated to individual vials for at least 24 hours prior to the assay. At the time of the assay one male of each population identity and colour was mouth aspirated into an empty vial, then flipped into a vial containing an acclimatised female. Mating latency (time to amplexus formation), colour of the mated male and mating duration were recorded. Under the conditions we used, copulations lasted on average between 15-20 minutes. A viable mating was considered one that lasted for more than two minutes, and vials in which mating did not occur within 60 minutes were discarded. This represents a commonly employed design to test mate choice (Dukas 2005, Ghosh & Joshi 2012, Arbuthnott *et al.* 2017). Vials were randomised so that observers were unaware of female identity. Trials were colour balanced so that in half of the trials the ACO male was blue and the CO male red, and in the other half the alternate dye pattern was applied. Flies were 2-3 days old at the time of the assay.

This assay was repeated over two days, with female choice tested in both a morning (9am-12pm) and afternoon (1pm-4pm) trial each day. In each of the 4 trials, we tested the choice of 50 females from both selection regimes for each of the 3 replicate populations. The

ratio of homotypic to heterotypic matings was calculated across the 50 replicate vials for each combination of replicate population and selection regime.

Male mate choice assay

It is difficult to distinguish the influence of male competition and courtship effort from female preference and resistance. By testing individual male mate choice in addition to female choice and contrasting the results, we hoped to uncover the source of any observed non-random mating pattern. From the time of virgin collection, males were maintained at 10 males/vial. Two females, one from each selection regime, were acclimatised to the same vial 24 hours prior to the assay. Female flies from ACO and CO selection treatments could be identified reliably based upon size alone. On the day of the experiment, one male was “pootered” (mouth aspirated) into a vial containing two females. As with the female choice assay, observers recorded mating latency, the size of the mated female (large or small) and mating duration and all other procedures were the same.

Group mate choice assay

To test for the existence of premating reproductive isolation between ACO and CO populations when mating occurred in a group environment, we evaluated female mate choice in vials containing 10 females and 12 males from each regime. Flies were handled and marked using the procedures outlined above for individual assays. On the day of the assay, male vials of paired ACO and CO populations were combined and then flipped into a female vial. Vials were observed until at least 8 (out of a possible 10) simultaneously copulating pairs were observed, and were then frozen for 60 minutes without disruption to the copulating pairs. The frozen pairs were observed, and the colour of each mated male was recorded. This methodology was repeated over two trials. During each trial, 10 vials were tested for each regime within each replicate population.

Hatchability

In order to determine the existence of postmating prezygotic isolation we compared the hatch rate of eggs produced from F1 and F2 crosses $CO_i♀ \times ACO_i♂$ and $ACO_i♀ \times CO_i♂$ as well as the parental crosses $CO_i♀ \times CO_i♂$, and $ACO_i♀ \times ACO_i♂$ ($i = 1,3,5$). Crosses were performed in vials with 12 virgin females and 10 virgin males. A period of at least 4 hours was allowed for mating and egg laying. From each cross, male and female progeny were collected as virgins and maintained in sex specific vials. Hybrid and parental crosses were staggered in an attempt to synchronise time of eclosion.

For tests of hatchability, 100 flies of each sex were deposited in a collector bottle with a plate of yeasted food. After an egg laying period of 3 hours the plate was removed. Eggs were transferred into vials using fine brushes and egg collection solution (Ashburner 2005). Each vial contained 90 eggs arranged in a grid pattern to facilitate easy counting. 9 such vials were created for each cross identity, for each of the three replicate populations. 24 hours after egg collection, the proportion of hatched eggs was determined. Vials continued to be maintained under standard conditions to be used in tests of viability, development time, and size.

Larvae to adult viability

To uncover any survival differences, we compared the larvae to adult viability of hybrids compared to flies from parental crosses. Twelve days after egg collection, the number of eclosed adults in each vial was recorded. The difference between the number of viable eggs and eclosed adults was used as a measure of larvae to adult viability. 9 vials were tested for each cross identity in each replicate population.

Development time

Development time of flies from hybrid and parental crosses was also analysed as an indicator of hybrid viability. Each vial was observed for eclosion over a period of 4 days,

beginning 24 hours prior to the time of expected peak eclosion for each line. For the first 2 days, vials were observed every 6 hours. For the following 2 days, observations were made every 12 hours. During each observation, the number of eclosed flies was recorded. An average eclosion time was then calculated for the vial. 9 vials were tested for each cross identity across each replicate population.

Body Size

Body size was measured using dry weight as a proxy. During peak eclosion, 20 female flies were collected from each cross identity. These flies were given 1 hour to physically mature and then were frozen. Flies were divided into 4 groups of 5 females and placed into tinfoil dishes to be dried in an oven at 70 degrees Celsius for 24 hours. Each dish was measured in a microbalance four times: twice with the flies and twice without. These totals were averaged and divided by 5 to provide an estimate of the dry weight of an individual fly.

Fertility - Competitive reproductive fitness

An adult fitness assay was completed to test for parental line and hybrid fertility differences. In this assay, red-eyed focal flies competed with control flies marked with a recessive brown-eye marker (bw^1) derived from the baseline. The IV_b population was selected as these flies are a common ancestor to both the focal populations, also having an intermediate life-history selection protocol. The recessive nature of bw^1 allowed the proportion of red eyed progeny to be used as a metric of paternity share.

Focal F1 and F2 flies of the hybrid crosses $CO_i♀ \times ACO_i♂$, $ACO_i♀ \times CO_i♂$ as well as the parental crosses $CO_i♀ \times CO_i♂$, and $ACO_i♀ \times ACO_i♂$ ($i = 1,3,5$) were tested. Crosses were performed in vials with 12 virgin females and 10 virgin males. A period of at least 4 hours was allowed for mating and egg laying. From each cross, male and female progeny (focal flies) were collected as virgins and maintained in sex specific vials at a density of 10 flies/vial.

Focal flies were anaesthetized under CO₂ and combined with four IV_b flies of the same sex (competitors) and four IV_b flies of the opposite sex (potential mates). Vials were supplemented with a sparse amount of yeast. Female focal and IV_b flies were acclimatised to vials 24 hours prior to the assay. After 48 hours, flies were flipped into new vials where oviposition occurred. Flies were 5-7 days old at the time of the assay. After 18 hours flies were discarded. At the time of eclosion, the number of red eyed and brown eyed offspring produced from the competition assay were counted. Thirty vials for each sex and cross identity were tested for each of the 3 replicate populations.

Statistical Analysis

Statistical analysis was conducted in R version 4.2.3 -- "Shortstop Beagle" (R Core Team 2023). Data was visually assessed for residual normality and heterogeneity. Where generalised linear models were used, residual dispersion was tested using the DHARMA package (Hartig 2022).

Female, male and group mate choice data was analysed with a repeated G-test for goodness of fit (Ghosh & Joshi 2012, McDonald 2014). In each case, the ratio of homotypic to heterotypic matings was tested for deviation from a 1:1 null expectation separately for each replicate population and overall for each selection regime. To study the effect of the male and female identity on mating latency and duration, we used linear mixed effects models (LMMs) fit with mated male identity and mated female identity as fixed effects and trial ID and replicate population as crossed random effects. Data from female choice and male choice experiments were analysed independently. In both experiments, mating latency models with raw data produced skewed residual distributions. To correct for this, we used mixed linear models using $\log(\text{mating latency} + 1)$ as the response variable.

To determine the effect of cross identity on fly development time and body size, LMMs were fit with cross identity as a fixed effect and replicate population as a random

effect. To determine the effect of cross identity on hatchability, viability and the fertility of flies, binomial generalised linear mixed effects models (GLMMs) were fit with cross identity included as fixed effect and parental replicate included as a random effect. Fertility data was analysed separately for males and females. Due to over-dispersion issues, we remodelled the viability data and the fertility data for both sexes with beta-binomial generalised linear mixed effects models using the 'glmmTMB' package (Brooks *et al.* 2017).

Models were fit using the 'lme4' package (Bates et al. 2015). Fixed effects and random effects were analysed using 'anova()', 'ranova()', 'Anova()' (Kuznetsova *et al.* 2017, Fox & Weisberg 2019). Post-hoc comparisons between cross identities were performed using the 'emmeans' package (Lenth 2022).

RESULTS

Female Mate Choice Assay

Overall, ACO females showed a significantly higher proportion of homotypic (0.57 ± 0.04 , mean $\pm 1.96 \times \text{se}$) compared to heterotypic matings (0.42 ± 0.04 , $P = 0.01$, fig 4.1a), with no significant heterogeneity among replicate population pairs ($p = 0.66$, table C1a). The ACO₅ population showed a significant deviation from the null hypothesis, with more homotypic matings compared to heterotypic matings ($p = 0.04$; fig C1a). Females from the ACO₃ population showed a similar trend towards more homotypic matings with borderline significance ($p = 0.05$). ACO₁ females showed no significant difference between mating types ($p = 0.26$). When data was pooled across trials within each replicate population, both ACO₃ and ACO₅ populations, but not the ACO₁ population, showed a significant deviation from a random mating ratio ($p_1 = 0.21$, $p_3 = 0.02$, $p_5 = 0.02$). with no significant heterogeneity between individual trials for any of the 3 replicate populations ($p_1 = 0.30$, $p_3 = 0.28$, $p_5 = 0.21$).

Overall, CO females showed a significantly higher proportion of homotypic (0.60 ± 0.05) compared to heterotypic matings (0.40 ± 0.05 , $p < 0.0001$, fig 4.1a), with no significant heterogeneity among replicates ($p = 0.65$, table C1a). Two CO populations, CO₁ and CO₃, showed an overall significant deviation from the null hypothesis, with more homotypic copulations ($p_1 < 0.01$, $p_3 = 0.01$, Figure S1a). CO₅ females showed a trend towards more homotypic matings, but this was not significant ($p = 0.08$). When data was pooled across trials, all three CO populations showed a significant deviation from the null hypothesis of random mating ($p_1 < 0.001$, $p_3 = 0.01$, $p_5 = 0.04$), with no significant heterogeneity among trials for any of the replicate populations ($p_1 = 0.13$, $p_3 = 0.15$, $p_5 = 0.28$).

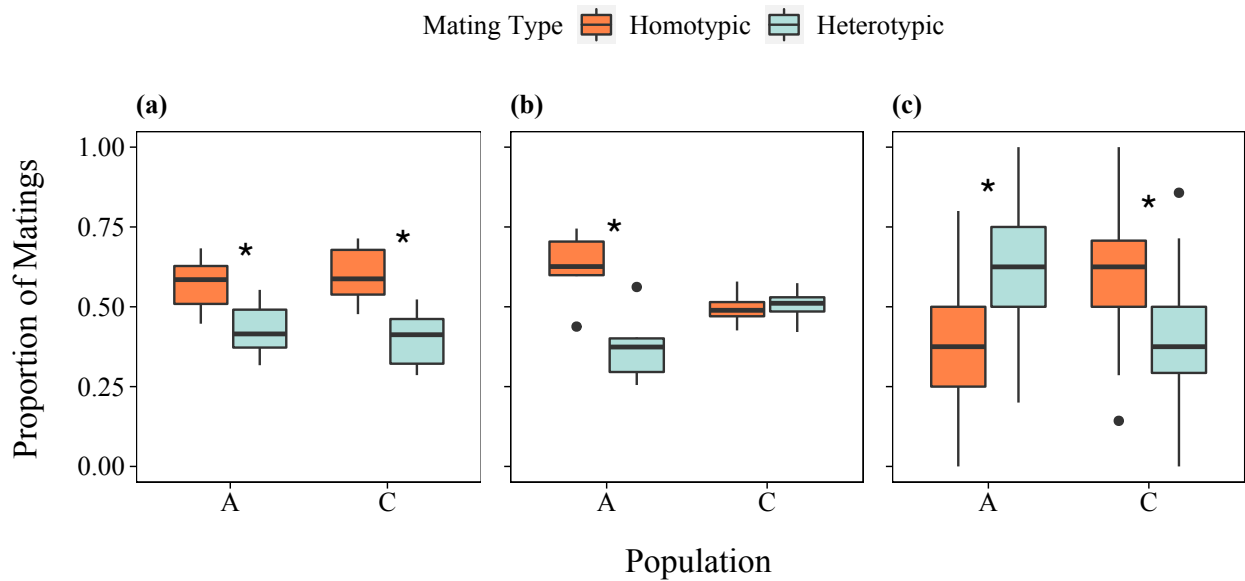


Figure 4.1. The proportion of homotypic compared to heterotypic matings recorded during the female (a), male (b) and group (c) mate choice assay. Female (a,c) and male population identity is shown on the x-axis.

Only mated male identity had a significant effect on mating latency ($p < 0.0001$, table C2). The crossed random effects of the replicate population and trial were significant ($p < 0.0001$), but the cumulative proportional variance attributed to the random effects of replicate population and trial was minimal (0.05). Both ACO and CO females displayed significantly shorter latencies in mating with CO males (11.8 \pm 1.2 min, 11.6 \pm 1.0 min respectively), compared to ACO males (15.3 \pm 1.3 min, 15.5 \pm 1.5 min respectively, fig 4.2a). There was no significant effect of female identity or interaction between male and female identity.

Female identity and interaction between male and female identity had significant effects on mating duration ($p < 0.0001$, table C2). The crossed random effects of the replicate population and trial were significant ($p < 0.0001$), but cumulative proportional variance attributed to the random effects of replicate population and trial was minimal (0.04). In general, ACO females (15.0 \pm 3.8 min) displayed shorter mating durations than CO females

(16.7 +/- 4.2 min), and both females mated longer with homotypic partners (ACO♀-ACO♂ 15.4 +/-0.4 min, ACO♀-CO♂ 14.3 +/- 0.5 min, CO♀-ACO♂ 16.1 +/-0.6 min, CO♀-CO♂ 17.1 +/- 0.4 min). All pairwise contrasts were significant (fig 4.2c) with the exception of ACO females mated with ACO males vs CO females mated with ACO males. There was no significant effect of mated male identity.

Male Mate Choice Assay

Overall, ACO males showed a significantly higher proportion of matings with ACO females (0.64 +/- 0.05) compared to CO females (0.36 +/- 0.05, $p < 0.0001$, fig 4.1b), with no significant heterogeneity between populations ($p = 0.81$, table C1b). All three A populations showed an overall significant deviation from the null hypothesis, displaying more homotypic compared to heterotypic matings ($p_1 < 0.0001$, $p_3 = 0.01$, $p_5 < 0.001$). There was significant heterogeneity observed among trials within the ACO₁ population ($p = 0.01$), but not the ACO₃ ($p = 0.70$) or ACO₅ ($p = 0.38$) populations.

Overall, CO males displayed a nearly equal proportion of matings with ACO (0.51 +/- 0.02) and CO females (0.49 +/- 0.02, $P = 0.95$, fig 4.1b), with no heterogeneity among replicate populations ($p = 0.93$, table C1b). All three CO populations showed no significant deviation from the null hypothesis of random mating ($p_1 = 0.99$, $p_3 = 0.65$, $p_5 = 0.96$). There was also no significant heterogeneity among trials within any of the replicate populations ($p_1 = 0.97$, $p_3 = 0.54$, $p_5 = 0.91$).

None of male identity, female identity or interaction between male & female identity influenced mating latency (table C2). The crossed random effects of the replicate population and trial were significant ($p = 0.01$), but cumulative proportional variance attributed to the random effects of replicate population and trial was minimal (0.02). Females from the CO populations displayed a slightly higher mating latency with both ACO and CO males (18.0

+/-1.7 min, 18.1 +/- 1.6 min respectively), compared to the ACO females (16.6 +/- 1.1 min, 16.9 +/- 1.4 min respectively) but these differences were non-significant (fig 4.2b).

Only mated female identity had a significant effect on mating duration ($p < 0.0001$, Table C2). The crossed random effects of the replicate population and trial were significant ($p < 0.0001$), but cumulative proportional variance attributed to the random effects of replicate population and trial ID was minimal (0.04). Males from the both populations displayed a slightly higher mating latency with CO females (16.3 +/- 0.7 min, 17.3 +/- 0.6 min respectively), compared to the ACO females (14.8 +/- 0.5 min, 15.0 +/- 0.7 min respectively) (fig 4.2d). There was no significant effect of male identity or interaction between male and female identity.

Group Mate Choice Assay

Overall, ACO females showed a significantly higher proportion of matings with CO males (0.62 +/- 0.05) compared to ACO males (0.38 +/- 0.05, $p < 0.0001$, fig 4.1c), with significant heterogeneity being observed between replicate populations ($p = 0.01$, table C1c). ACO₃ and ACO₅ females showed an overall significant deviation from the null hypothesis, displaying more heterotypic compared to homotypic matings ($p_3 = 0.02$, $p_5 < 0.0001$). ACO₁ females showed no deviation from the null expectation ($p = 0.78$). There was no significant heterogeneity observed among trials within any of the three replicate ACO populations ($p_1 = 0.49$, $p_3 = 0.45$, $p_5 = 0.50$).

Overall, CO females showed significantly more homotypic (0.61 +/- 0.04) compared to heterotypic (0.39 +/- 0.04) copulations ($p < 0.0001$, fig 4.1c), with no heterogeneity between replicate populations ($p = 0.12$, Table C1c). CO₅ females showed an overall significant deviation from the null hypothesis, with more homotypic compared to heterotypic copulations ($p < 0.001$). CO₁ and CO₃ populations showed no overall deviation from a random mating ratio of 1:1 ($p_1 = 0.10$, $p_5 = 0.07$). When data was pooled across trials, both

CO₃ and CO₅ populations showed significant deviation from a random mating ratio ($p_3 = 0.02$, $p_5 < 0.0001$). There was no significant heterogeneity among trials within any of the populations ($p_1 = 0.10$, $p_3 = 0.58$, $p_5 = 0.96$).

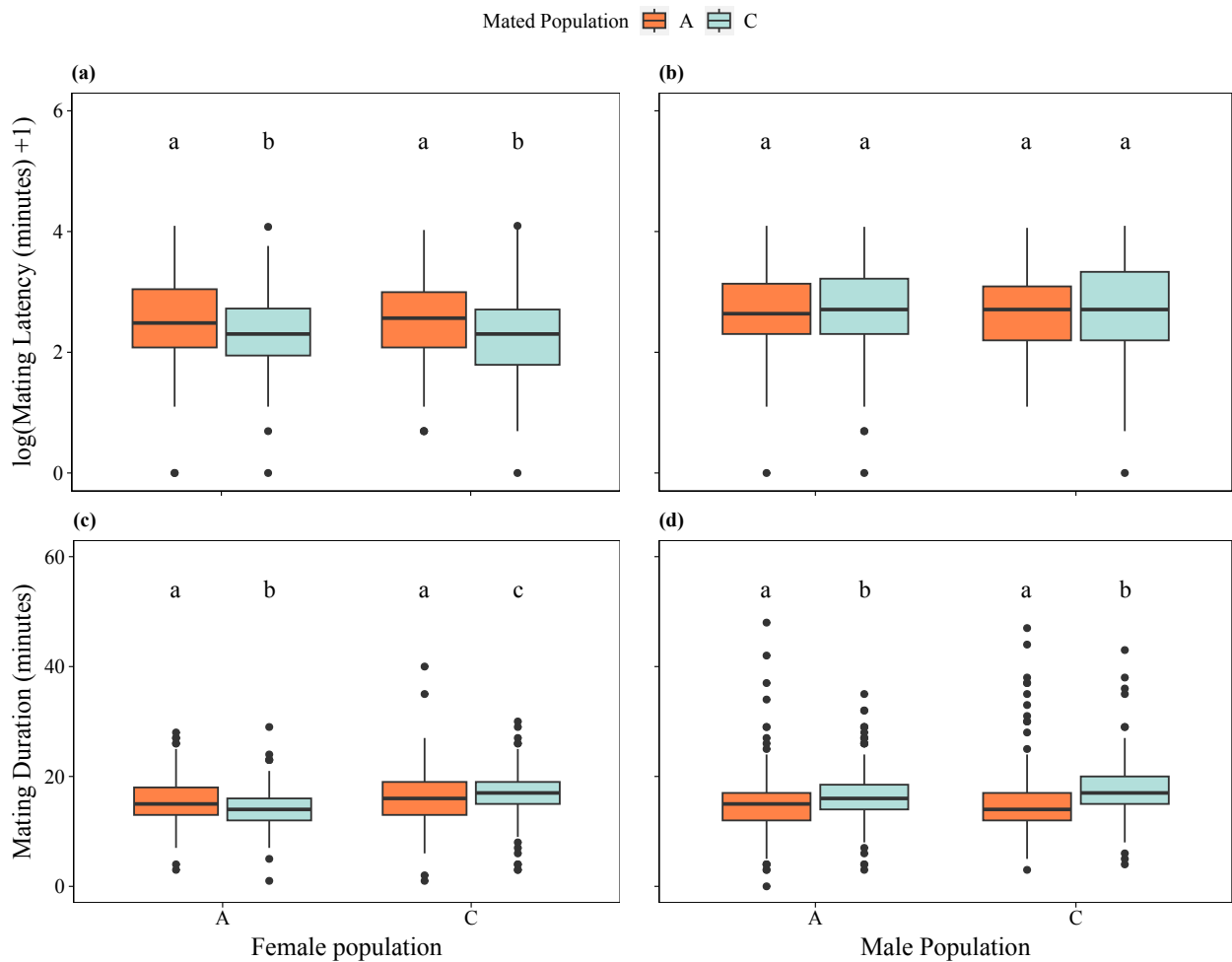


Figure 4.2. The latency (a,b) and duration (c,d) of matings in the choice assays. Female (a,c) and male (b,d) population identity is shown on the x-axis. Letters indicate significant differences between mating types.

Post-mating prezygotic reproductive isolation

Hatchability

Cross identity had a significant effect on egg hatchability ($p < 0.01$, table C3). The random effect of replicate population was not significant ($p = 0.89$). The only significant differences in hatch rate was the decline of $CO_i\text{♀} \times A_i\text{♂}$ F2 hybrids compared to ACO and CO parental lines (fig 4.3a). Overall, hatchability was high with average values between 0.96 \pm 0.01 ($CO_i\text{♀} \times A_i\text{♂}$ F2 hybrids) and 0.98 \pm 0.01 (ACO parentals).

Postzygotic reproductive isolation

Larvae to adult viability

There was also a significant effect of cross identity ($p < 0.0001$, table C3), but no significant random effect of replicate population ($p = 0.31$) on the viability of flies. On average, larvae to adult viability was lowest for ACO parental (0.78 \pm 0.03) and highest for $CO_i\text{♀} \times ACO_i\text{♂}$ F2 flies (0.93 \pm 0.02). Both $CO_i\text{♀} \times ACO_i\text{♂}$ hybrids and $ACO_i\text{♀} \times CO_i\text{♂}$ F2 hybrids demonstrated significantly higher viability than ACO, CO and $ACO_i\text{♀} \times CO_i\text{♂}$ F1 flies (fig 4.3b).

Development time

There was a significant effect of cross identity ($p < 0.0001$, table C4), but no significant random effect of replicate population ($p = 0.51$) on the development time of flies. All four hybrid crosses demonstrated development times that were significantly longer than ACO parental flies (175.76 \pm 0.5 hours) and significantly shorter than CO parental flies (218.32 \pm 1.2 hours). There were no significant differences in development times among the four hybrid crosses (fig 4.3d).

Body size

Cross identity also had a significant effect on the body size of flies ($p < 0.0001$, table C4). The random effect of the replicate population was not significant ($p = 0.23$). All four

hybrid lines showed average individual weights that were significantly higher than parental ACO lines (0.21 +/- 0.01 g), and significantly lower than parental CO lines (0.31 +/- 0.01 g). Hybrid $ACO_i\text{♀} \times CO_i\text{♂}$ F1 flies were also significantly heavier than $ACO_i\text{♀} \times CO_i\text{♂}$ F2 flies (fig 4.3c).

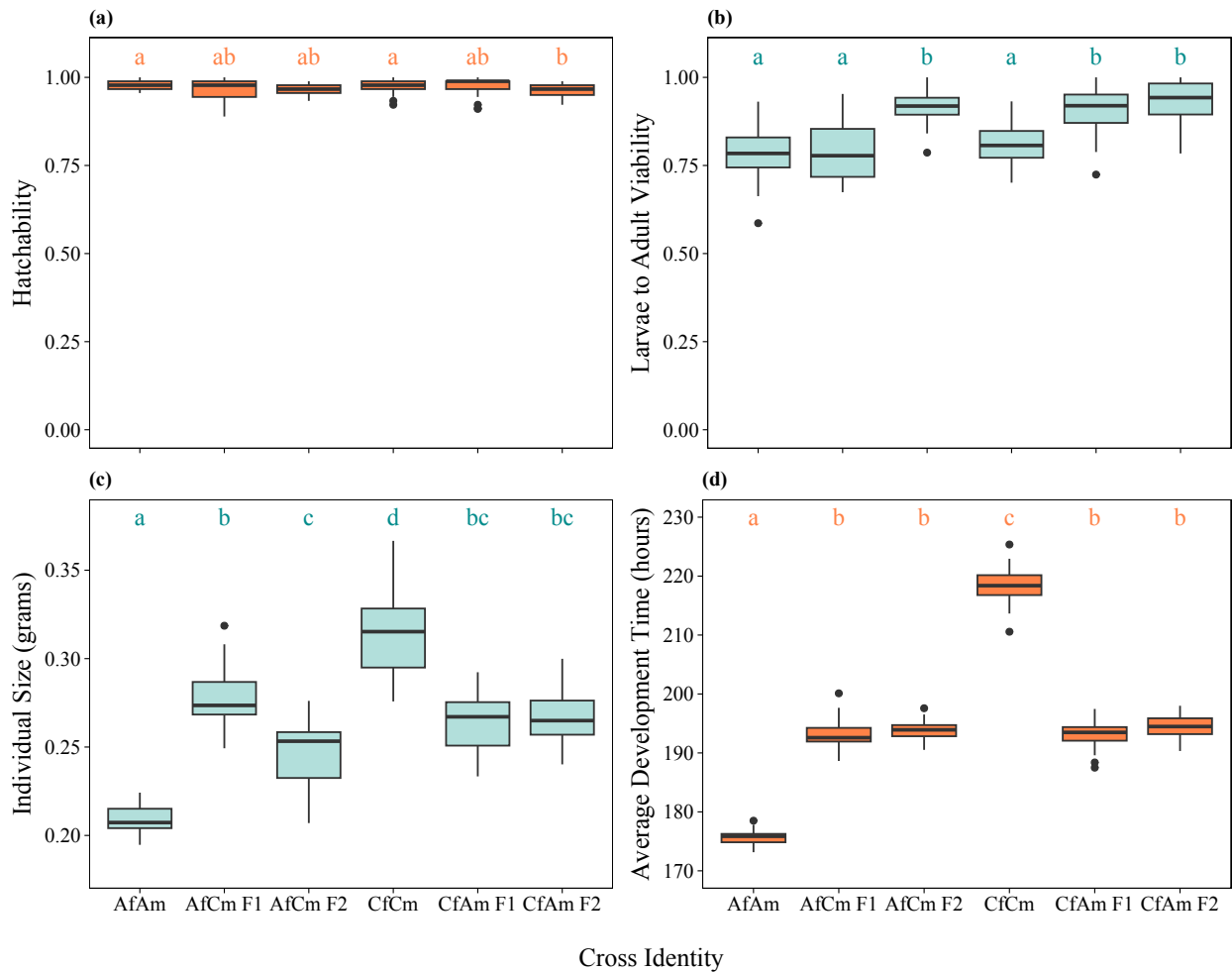


Figure 4.3. The hatchability (a), egg to adult viability (b), body weights (c) and average development time (d) of flies from parental and hybrid crosses. Letters indicate significant differences between cross identities.

Reproductive fitness

Cross identity had a significant effect on female reproductive fitness ($p < 0.0001$, Table C5). Parental CO lines showed the highest average fitness (0.24 ± 0.03), parental ACO lines the lowest (0.12 ± 0.02) and hybrids displayed intermediate fitness between parental lines (fig 4.4). All cross identities produced significantly more offspring than parental ACO females and flies from all cross identities, except for $CO_i \text{♀} \times ACO_i \text{♂}$ F1 females, produced significantly fewer offspring than parental CO flies. In both cases, second generation (F2) females displayed lower reproductive fitness than first generation (F1) hybrids, but this effect was only significant for the $CO_i \text{♀} \times ACO_i \text{♂}$ crosses. There was no significant effect of population replicate on female fertility ($p = 1$).

There was a significant fixed effect of cross identity ($p < 0.01$, Table C5) but not replicate population ($p = 1$) on the fertility of male flies. $CO_i \text{♀} \times ACO_i \text{♂}$ F1 male hybrids showed the highest average fitness (0.13 ± 0.04) and ACO males the lowest (0.05 ± 0.02). CO and $CO_i \text{♀} \times ACO_i \text{♂}$ F1 males demonstrated a significantly higher fitness than ACO males (fig 4.4). Similar to that observed in the female assay, male F2 hybrids displayed lower fitness than F1 hybrids, but these differences were not significant. While female flies displayed fitness values around the expected value, males of both parental and hybrid cross identities produced a lower proportion of offspring than expected when in competition with four competitors.

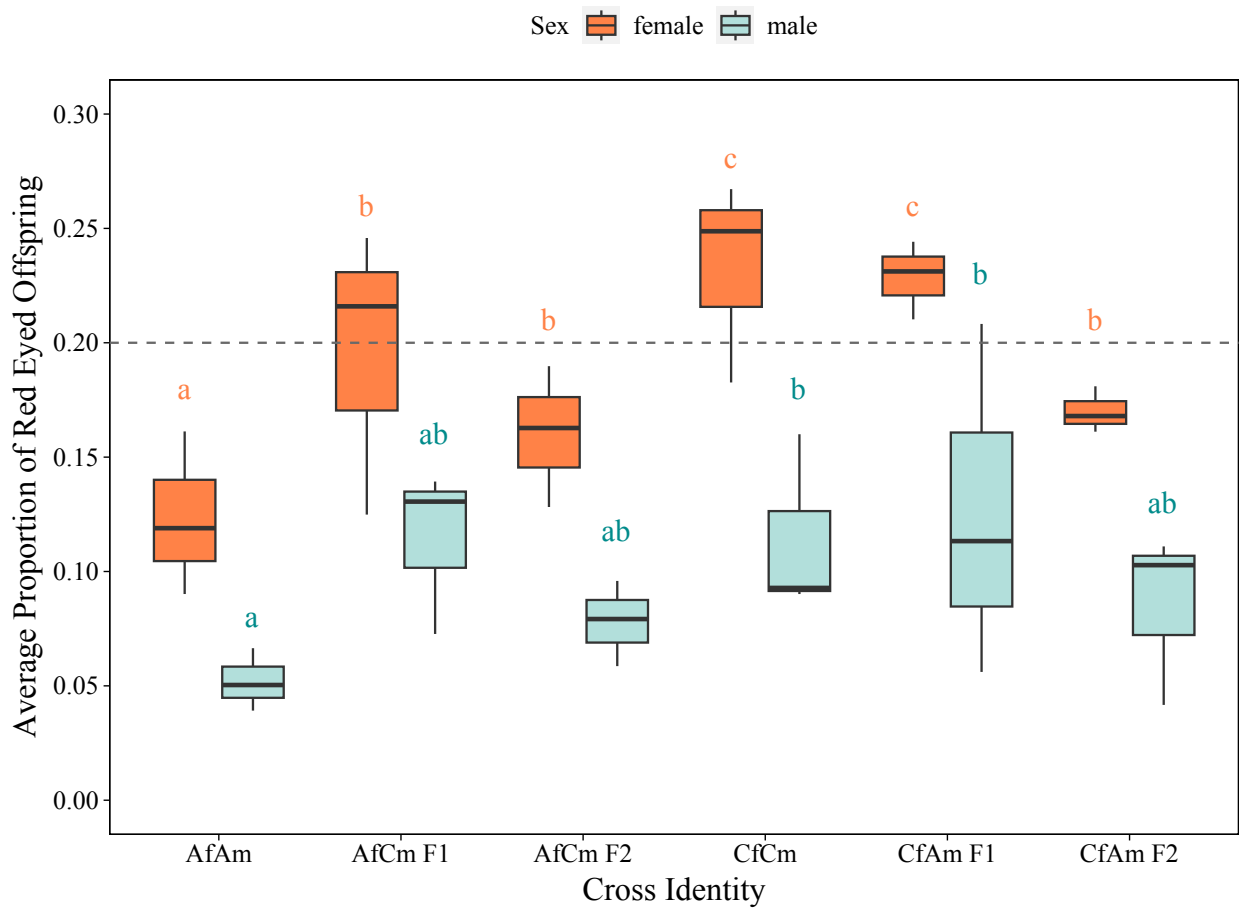


Figure 4.4. The average proportion of red eyed offspring produced by flies from parental and hybrid crosses, in competition with brown eyed competitors. Average proportions were determined for each replicate population and plotted. The dashed line indicates the expected proportion of $\frac{1}{5}$ of the offspring. Letters indicate significant differences between cross identities.

DISCUSSION

Our experimental assays show the striking differentiation between the ACO and CO populations after over three decades and 1,500 generations of divergent selection on demography. The ACO population adults eclose nearly two days earlier than the CO population adults do. The clear tradeoff between speed and size is evident, as A adults are about 33% lighter than CO adults, and this is likely related to the strong differences in adult fitness we observed between the two selection treatments. Interestingly, the tradeoff between developmental speed and juvenile viability (Chippindale et al. 1997) is not apparent here or in other recent unpublished data on these populations, suggesting compensatory evolution over time. Given these differences in morphology and life history, we asked if we could detect the early stages of the speciation process for these populations in allopatry under different ecological circumstances.

To summarise our results, we found evidence for the evolution of premating barriers to gene flow between these diverged populations. Mate-choice assays generally reflected positive assortative mating patterns driven by female mate-choice. Females from both focal populations showed strong homotypic mate choice in individual tests, but this pattern was less clear in group tests, particularly as the smaller ACO females tended towards heterotypic mates. Under conditions of female choice, mating durations of homotypic pairs were consistently longer than heterotypic pairs. Despite this (and homotypic mate choice), the larger CO males showed lower latencies to mating with both females. In contrast, under conditions of male choice (no male-male competition), ACO and CO males showed no differences in mating latency. Further, under male choice conditions, both males mated for longer durations with the larger CO females, suggesting sperm catering based on body size rather than homotypic preference. We show that post-mating prezygotic barriers have not evolved to the point of detectability between these populations in the form of gamete

incompatibility, measured through egg hatchability. We also find no evidence of postzygotic reproductive isolation in the form of survival to reproductive maturity, and measurements of development time and body size show that hybrids display intermediate trait values relative to the parental treatments. We observed only suggestive trends for postzygotic reproductive isolation in the form of hybrid breakdown in competitive reproductive fitness assays. F_1 hybrids display intermediate fitness to that of the two parentals, however, there is a tendency for F_2 hybrid offspring to display lower fitness than the F_1 hybrids; while potentially an indicator of evolving Dobzhansky-Muller incompatibilities, our data are merely suggestive at this juncture, meriting further investigation.

It has been suggested that prezygotic isolating barriers will typically evolve before postzygotic barriers, and *Drosophila* seems to adhere to this rule (Arthur & Dyer 2015). Within the category of prezygotic barriers, premating RI is expected to evolve before postmating-prezygotic RI (Turissini et al. 2018). Our data are generally in accord with these predictions, as our diverged population pairs show marked premating reproductive isolation, at least under individual choice tests, but clear evidence for later stage RI is lacking.

Strong divergence in body size exists between ACO and CO animals (Xiao et al. 2019; data herein) which is presumed to correlate with differences in persistence/resistance capabilities between the selection treatments. This size difference led us to expect the presence of some premating reproductive isolation. However, we predicted premating isolation in one direction, on the basis of greater resistance from larger (CO) females, greater persistence of CO males, and a possible preference for large mates. That directional RI was noted by Ghosh & Joshi (2012) in a study similar to ours: bigger mates were preferred by both large/slow and small/fast flies. Contrary to that precedent, females of both regimes displayed strong homotypic mate choice and longer mating durations with their homotypic partners in individual tests. This result is especially intriguing for the ACO females, as they

displayed a preference for smaller ACO males even though *D. melanogaster* females are expected to prefer larger males (Pitnick 1991, Jagadeeshan et al. 2015, but see Prasad et al. 2007).

Male mate choice tests may present trickier interpretations. ACO selected males largely mated with homotypic females, but male COs mated nearly randomly. While ACO males appear to be showing positive assortative mating preferences, both types of males were observed making repeated courtship attempts with both homotypic and heterotypic females (unquantified observations) suggesting that the skewed mating pattern could be the result of the increased capability of CO females to resist unwanted mating attempts, particularly from the smaller A males. In alignment with this hypothesis, we observed a non-significant trend of longer mating latencies with CO females. In populations of flies evolved under a similar pair of life history selection regimes, Ghosh and Joshi (2012) found that small males attempted to court large females, but were unsuccessful, and, as a result, mated more frequently with small females. We therefore cannot easily ascribe mating assortment to the choice of the male, who may be indiscriminating under the test conditions, particularly with no competition for mates. Concurrent with this, we note no differences in the mating latencies of the two male types in the absence of male-male competition. ACO-type females are also under tremendous pressure to mate, as they are selected to lay eggs about 24h after becoming sexually mature. In the absence of competition, many males may have simply taken the most receptive mate first; for the CO males this may have counterbalanced a homotypic preference, creating the ambivalent outcome observed. The results of the mate choice assays taken together suggest that premating isolation in this system is most strongly driven by female choice.

Body size is a likely trait involved in the evolution of reproductive isolation between the population pairs tested. However, it remains possible that the patterns of assortative

mating stem from traits that are yet to be characterised. Some possibilities include courtship behaviours (mating call, wing movements) and cuticular hydrocarbon profiles that may have diverged under the divergent selection regimes. Besides female preference, body size differences also play a clear role in the differential capacities displayed by females to resist mating attempts by males, which creates a further reproductive barrier between large females and small males. Despite large body size differences between the selection treatments, it seems unlikely that the patterns of positive assortative mating can be explained by mechanical incompatibilities in copulation alone; some earlier anecdotal observations suggested they may exist, but we saw no clear evidence in these trials.

In the group mate choice assays, female CO flies continued to show homotypic mating preference, but A females displayed reversed preferences, mating more frequently with heterotypic males than homotypic males. The existence of heterotypic mate choice in groups, in spite of homotypic mate choice in individual assays suggests that the nature of the assay plays a role in the mating patterns observed. In the group mating assays, vials were more crowded (34 individuals vs. 3) - potentially creating circumstances where resisting forcible mating-attempts is more difficult for females. This is a likely explanation given that only the small A females displayed reversed mate choice preferences, while the larger CO females largely reproduced the same mate choice patterns as displayed in individual assays. It is worth testing if similar mate choice assays conducted in larger arenas (such as 90mm petri dishes) would reverse this preference to homotypic choice. At the time these experiments were conducted (since changed) there was an important difference in the mating environment of the ACO and CO selection treatments: whilst the early-reproducing ACO's primarily mated in vials, with only a brief phase in cages for egg-laying, the CO's, with 14 days in cages probably do all mating relevant to fitness in the cage environment. With these differences in the "natural" mating environment of our LTEE, the mate choice patterns found

in individual assays seem to show us that A-selected females have a preference for homotypic mating but are unable to exercise it in a group mating setting with larger males.

Asynchronous development time alone can contribute to reproductive isolation, as seen in the experimental work of Miyatake and Shimuzu (1999) on the melon fly. Because the mating times diverged in lines selected for development time, significant RI arose that was evidenced in experimental mate choice trials. Similarly, the extreme early reproduction regime of the A populations means that neither CO individuals nor A/CO hybrids could hybridize, as the latter groups would not even be sexually mature before a generation is completed. This scenario is, of course, artificial because natural populations generally do not grow on synchronised, discrete generations. they are not directly responsible for the assortative mating patterns observed in the controlled mate-choice assays.

Post-mating, we report no evidence of reproductive isolation deriving from gamete incompatibility, as would be evident in reduced egg hatch rates. Similarly, our measurements of larva to adult viability data show that there are no hybrid treatments with lower levels of viability than the purebred parental populations. In fact, we observed trends of hybrid vigour, with some of the hybrid treatments displaying higher larva to adult viability than the parental treatments. Interestingly, the larva to adult viabilities in F2 treatments were significantly higher than that of the parental selection treatments and one F1 treatment. While the increased viability in hybrids could suggest the existence of homozygous deleterious variation in developmental loci in our selection regimes, this should be consistently seen in both F1 and F2 generations, instead of being more common in the F2 generation. Development time and body size displayed no suggestion of abnormalities indicative of developmental instability, with intermediate hybrid trait values relative to the parental treatments in both sexes.

The competitive reproductive fitness of hybrids was generally intermediate to the parental treatments, and we found no evidence of hybrid sterility. Given that both assays subjected a focal fly to 4 competitors of the same sex, we expect focal flies to sire/dam $\frac{1}{5}$ th of the offspring produced, if all else was equal. In female reproductive fitness assays, our populations did cluster around the predicted threshold, with hybrids scoring intermediate fitness levels relative to the low fitness ACOs and high fitness COs. In male reproductive fitness assays though, fitness fell well below the predicted threshold in all treatments. We rule out the possibility of this being a case of Haldane's rule, because male fitness falls well below predicted levels in parental males as well as hybrids. We believe that this is a consequence of the usage of IV_b flies as competitors/mates in these assays. Selected as common ancestors to both selection regimes, the IV_b have evolved in allopatry with both the populations, with a slightly different maintenance regime. We believe that the reduced male fitness estimates may be a consequence of homotypic mate choice by IV_b females in male fitness assays. While we do see different levels of competitive reproductive output for the different treatments in the female assay, it is not possible to disentangle male mate choice from innate fecundity and harm resistance differences in the females of different selection regimes. Overall, in spite of the depressed male fitness output relative to predictions, hybrids in both sexes still consistently showed intermediacy in fitness, suggesting no reproductive sterility and a link to an additive trait such as body size.

The comparison between F1 and F2 reproductive fitness revealed an interesting trend: F1 fitness was consistently higher than that of F2 offspring, however, this difference was statistically significant in only 1 out of 4 comparisons (2 reciprocal crosses *2 sexes). We interpret this with caution as a possible trend of hybrid breakdown as a consequence of incompatible gene complexes evolving in the two selection regimes.

We generally found high repeatability across our three independent replicates, which suggests that the characters measured and pre-mating RI observed stem from consistent treatment differences between the CO and ACO populations. Internal random processes of divergence in allopatry, such as the coevolution of arbitrary signaller-receiver relationships in allopatry may also drive the evolution of RI. A system like ours opens the door to a potential test for such divergence if RI were investigated between replicate populations *within* selection treatment, rather than *between* treatments. Although our three-decade experiment seems too young to see more than hints of post-mating RI, more sensitive queries of characters like developmental stability (e.g., as measured by fluctuating asymmetry) may be of interest. Further investigation of the sources of pre-mating RI and its environmental sensitivity are warranted.

CHAPTER 5: CONCLUSION

You get exactly what you select for.

A speciation “life-history” event

A core concept in our understanding of the biological world is the notion of speciation – the evolutionary separation of organisms by the yardstick of reproductive isolation (RI). In the evolutionary timetable, or “life-history” of speciation (the basic canon thereof) – reproductive isolation is typically caused by divergent adaptation due to heterogeneity of ecological niche, reduced gene flow over long periods of time, or a combination of the two. In 2018, fresh out of my master’s, I was interested in the idea that sexual conflict was an “engine for speciation” (Gavrilets 2014) – a parameter that could erect a premating RI barrier without invoking long periods of allopatry or niche divergence. Specifically: Male-female coevolution in response to conflict over mating rate (inter-locus sexual conflict - IeSC) can result in cycles of adaptive changes in traits such as mating behaviours and paternity certainty; and rapid (but arbitrarily divergent) co-evolution at these loci in allopatric populations should produce premating reproductive isolation (RI) even given identical ecologies. Studies using monogamy / sex ratio manipulations had started to demonstrate evidence for this idea – increased opportunity for polyandry (and by extension IeSC) resulted in premating RI between populations maintained under identical laboratory conditions, within 200 generations of allopatry (Syed *et al.* 2017).

When I arrived at Queen’s at this time, I was presented with a fascinating experimental system – a long term evolution experiment (LTEE) designed to select *Drosophila melanogaster* populations in opposite directions for a life history trait – age at reproduction. After generations of active selection for early and late reproduction, replicate population pairs were now maintained on 9 (ACOs) and 28 day (COs) discrete cycles.

Amongst other adaptive changes, the flies had diverged substantially in body size, to the point of being distinguished by the naked eye through body size alone. This led us to predict a greater intensity of IeSC as a consequence of the increased body size in CO populations, through increased potential for mate harm and mate harm resistance. If true, what were the consequences of such an asymmetric change in IeSC intensity? For instance, could these changes produce asymmetric premating RI, where the larger COs alone display homotypic mating preferences? In addition, we expected the larger animals to be more attractive, due to a correlation with higher reproductive fitness. Could such a preference exist across these populations, giving the COs and ACOs homotypic and heterotypic preferences respectively?

In a similar selection experiment in *D. melanogaster*, Ghosh & Joshi (2012) had demonstrated such a result. However, rather than selection for age at reproduction causing adaptive changes in development time, their “early” populations had been subject directly to selection for rapid development time. These had revealed trade-offs not only with body size but also with locomotion and activity levels – drastically increasing the odds of premating RI. By contrast, the ACOs were visibly far from lethargic, and likely provided a more rigorous test for the evolution of asymmetric premating RI.

Now we already knew a little about where these populations were in terms of the RI timetable. In spite of substantial adaptive changes, they were capable of producing viable and fertile hybrids – indicating that they had not undergone postzygotic RI, or at least not much of it. Nevertheless, these populations had also been separated under divergent selection for longer than most LTEE (excluding bacterial, yeast systems) – allowing us to conduct an empirical test of whether this timeframe (~ 1,500 generations) was sufficient to cause bi-directional RI through divergent selection and allopatry.

Divergent selection under allopatry produces premating reproductive isolation

Working with Chloe Robinson & Adam Chippindale, I found evidence for bi-directional premating RI in mate choice assays conducted on CO and ACO flies. Females from both selection treatments displayed strong homotypic mate choice surveyed individually, but this was only true of the CO females in group scenarios. In the ACOs, homotypic female mate choice in spite of reduced body size provided us with clear evidence that RI had evolved as a consequence of niche divergence in allopatry.

While male COs displayed indiscriminate mating attempts, ACO males displayed strong homotypic preference. Homotypic “preference” in ACO males is likely due to a persistence-resistance mismatch with CO females, as a consequence of evolved differences in body size and IeSC intensity. This was echoed in mating latency and duration data. Under female choice conditions (polyandry), CO males displayed a lower latency to mate with females from either population, relative to ACO males, suggesting that ACO males were perhaps easier to rebuff in mating interactions. Both females spent longer periods mated with conspecific males. Under male choice conditions (polygyny), we found no differences in latency to mate. Both spent longer periods of time in copula with CO females.

Taken together, we found clear signs of premating RI evolving through homotypic female preference in response to divergent selection under allopatry, demonstrating that 1,500 generations is sufficient evolutionary time for incipient speciation. In addition to this, increased intensity of IeSC contributes an asymmetric premating barrier between small males and large females.

In addition to “timing” premating RI, we also showed that 1,500 generations was not sufficient time for any other form of RI to evolve. We found no postmating-prezygotic or postzygotic RI regardless of direction of cross, and even found trends of increased F2 hybrid

viability. F2 hybrids did display a trend of reduced reproductive fitness relative to F1 hybrids in both sexes, which could be an early sign of postzygotic reproductive isolation.

Correlated changes in IeSC and reproductive isolation

While increased opportunity for polyandry is expected to change intensity of IeSC and result in speciation through rapid and arbitrary local coevolution, can we expect the same to be true of IeSC intensity differences as a result of correlated changes to other selection pressures? An interesting follow-up to this work could ask if the differences between these selection treatments also results in varied levels of RI between replicate populations of each treatment, in the absence of niche heterogeneity. For instance, would we find greater levels of RI between CO_i and CO_j , as against ACO_i and ACO_j ? Previous work in a related population has demonstrated trends of female fitness reduction as a consequence of inter-replicate crosses (Long *et al.* 2006). Would such trends scale with body size and correlated changes in IeSC intensity?

Is intra-locus sexual conflict a capacitor for genetic variance?

Besides my interest in engines accelerating speciation, I was also interested in whether sexual conflict plays a role in the maintenance of balanced polymorphisms, contributing to heritable variance for fitness. The maintenance of genetic variance is central to the study of evolutionary biology. Evolutionary change occurs through the selection of heritable variants that are best suited to the environment imposing the selection pressure. Inherent in this process is the culling of heritable variants that are poorly suited, leading to a reduction of heritable variance. Populations that are capable of maintaining heritable variation in the form of balanced polymorphisms are more likely to be able to adapt to abrupt changes in ecological niche without risking extinction. However, selection does not act on populations, allowing them to adapt insurance policies against extinction. The evolution of

balanced polymorphisms instead relies on selective processes acting on individuals, resulting coincidentally in the maintenance of heritable variation.

In sexually reproducing populations, conflict over shared traits with different fitness functions in the two sexes (intra-locus sexual conflict - IaSC) may act as one such process. Very simplistically – two alleles could co-exist at a locus, with each beneficial to one sex while deleterious to the other. Although fascinated by this prospect, I found that empirical tests of this idea were incredibly hard to design. Ideally, this demanded a comparison of heritable variances before and after a surgical knock-down of IaSC that did not disturb the allele-shuffling capacities of sexual reproduction.

One possibility arrived at our doorstep, in the form of a departmental seminar. In a visit to Queen's, Karl Grieshop described a novel application of the diallel cross (a quantitative genetic design used to estimate components of trait variance), and its capacity to reveal sex-specific dominance reversals (SSDRs) (Grieshop & Arnqvist 2018). The evolution of sex-specific dominance reversal is a potential downstream outcome of IaSC, where alleles evolve to be dominant in the sex where they are beneficial to fitness and recessive in the sex that they are deleterious. By allowing populations to (partially) overcome the fitness load imposed by IaSC, SSDRs vastly expand the parameter space leading to stable balanced polymorphisms. The identification of such SSDRs in *D. melanogaster* would contribute indirect evidence for the role of IaSC in maintaining heritable variation. To date, we know of only one demonstration of SSDR in this species – in surviving against pathogen infection (Geeta Arun *et al.* 2021).

Weak links in the armour: Where should sexually antagonistic alleles invade the genome?

While SSDRs offer a mechanistic route to balanced polymorphisms, they were also frequently invoked to address a second open question about sexually antagonistic loci.

Alleles on the X chromosome are expressed in males by default, but can be recessive in the female, potentially evading selection until reaching a certain frequency in the population. However, if SSDRs are easily evolved – the X is no longer a privileged refuge for SA variation.

The diallel cross is capable of identifying X linked trait variance using sons derived from the two directions of a reciprocal cross. However, the process of inbreeding (required to create the lines used in a diallel) was likely to filter out a certain amount of recessive deleterious variation, making it less than ideal for our stated purpose. I recognized that for the X chromosome at least, we had access to a more powerful quantitative genetic design – the hemiclinal analysis of variance (Chippindale and Rice 2001).

Hemiclinal analysis promised the following: (1) the capacity to assess the standing genetic variance and intersex genetic correlations in our populations of interest, (2) the potential to tease apart X-linked and autosomal contributions to this variance and (3) a system to identify sexually antagonistic haplotypes; all without first mangling the standing genetic variance through inbreeding.

Indexing sexually antagonistic loci: An IaSC phonebook

Identifying SA haplotypes was through hemiclinal analysis offered an opportunity to identify genomic associations with IaSC. Besides providing valuable insights into the distribution and number of loci underpinning IaSC – the IaSC phonebook would have also allowed for comparative analysis. Do putative SA loci actually demonstrate greater nucleotide variability compared to the rest of the genome? Do these regions reveal signatures of balancing selection acting on the genome? Ruzicka *et al.* (2019) produced this exact finding, answering both questions in the affirmative while we were still drafting the necessary breeding designs. Even more impressively, Ruzicka *et al.* demonstrated that these putative

SA loci displayed increased variability and signatures of balancing selections in a distant African outgroup separated by ~ 1 million years. This is perhaps the first empirical demonstration of the maintenance of SA balanced polymorphisms.

Male-limited selection: a promising approach

In the meantime, having embarked on parallel projects creating diallelic and hemiclinal breeding designs, I had to shelve these projects to work within pandemic safety measures. Instead, I set up a male-limited (ML) selection experiment, designed to release haplotypes from selection through the female fitness function, without modifying genetic correlations between the sexes or shutting down genetic shuffling through recombination. ML selection was expected to fix SA male variants, improving male fitness at the cost of female fitness. These fixation events would reduce heritable trait variance, and masculinize both the genome and the phenotype. Although slower (and more laborious), this design offered a number of advantages in answering our questions of interest. (1) We could now test for a reduction in heritable variation for fitness in the absence of IaSC. (2) Quantitative genetic designs are likely to overestimate SA loci enrichment on the X chromosome (Connallon & Ruzicka 2020). Using a selection experiment instead, we could now address if the X chromosome was disproportionately responsible for SA fitness effects. Lastly, (3) we could study genomic associations with evolved changes between the selection treatments, improving the signal-noise ratio in indexing the IaSC phonebook relative to hemiclinal analysis.

Intra-locus sexual conflict obscured in a male-limited selection experiment

Working with Imran Sayyed, Mindy Baroody, Josh Kowal & Adam Chippindale, I found no generic improvement in male fitness as expected by IaSC theory, in a ~90 generation long male-limited (ML) selection experiment conducted on a novel base population.

Male fitness of selected haplotypes was consistently and significantly improved relative to controls under the conditions in which selection was carried out. This improvement was facilitated by local adaptation to a novel genetic background and arrested mates, with an interaction between the two factors. Teased apart, I was able to demonstrate that ML animals displayed improved male fitness when tested in the background of the cytotypes and Y chromosomes (both independently) from the selection experiment. While selection improved mating success and reduced mate harm, it did not affect sperm offense or fecundity induction. We detected no signature of sex chromosome drive.

In parallel, these haplotypes displayed reduced fitness relative to controls when expressed as females. This suggested either a sexually antagonistic (SA) basis for the improvement in male fitness, or that female fitness independently decayed as a consequence of relaxed selection on female-limited traits / loci. Male adaptation to the cytotype used in the selection experiment produced a correlated improvement in female fitness for the selected haplotypes, suggesting sexually concordant evolution in response to this source of selection. We did not test fitness effects of adaptation to the Y chromosome on females, as it is not a part of their typical genetic configuration.

Ancestral test conditions

In our attempt to characterise generic improvement in male fitness, a simple yet key factor that we overlooked was the potential confound of adaptation to a specific local environment. To assay this generic improvement in fitness, we chose to express selected haplotypes under ancestral conditions – intended to remove the selected animals from their peculiar genetic backgrounds. Previous work had demonstrated fitness costs attached to some cytogenetic constructs used to carry out this work (Abbott *et al.* 2013), and the evolutionary arrest of the selection conditions in the ML populations amplified the opportunity for narrow, locale specific adaptation. In chapter 3, I describe this through the use of “home court” and

“wild type” genetic backgrounds associated with selected and control populations respectively. Reciprocally though, the ancestral conditions that we tested fitness in was the home court for control populations. Here, instead of the risk of a spurious positive result, this confound potentially negated generic improvements in male fitness, and contributed to the consistent equivalency in male fitness between selected and control lines. This could have been avoided by introgressing both selected and control haplotypes into a third, neutral genetic background to assay fitness. Unfortunately, this did not occur to us at the time of experimentation.

Is the X chromosome a hotspot for sexually antagonistic variation?

Two previous sex-limited X selection experiments have revealed no sexually antagonistic fitness effects (Lund-Hansen *et al.* 2020, Abbott *et al.* 2020). While Lund-Hansen *et al.* find sexually concordant fitness effects for female-limited Xs (FL-X), Abbott *et al.* identify an improvement in one sex without changes in the other in response to the ML-X. Here, the ML experiment we conducted displayed an interesting pattern of X linked fitness effects. Comparing male fitness of ML_{SD} (ML-single dose) and ML_{SD(a)} (ML-single dose of autosomes) treatments, we found that the absence of the ML-X chromosomes in ML_{SD(a)} resulted in a nominal reduction of fitness (Tukey adj. $p = 0.11$). This relationship was more pronounced in 2 out of 3 replicate pairs, and completely absent from the third. Unlike previous selection experiments, this suggests a trend of male fitness improvement facilitated by the X chromosome. However, even here we do not demonstrate disproportionate X-linked fitness effects, merely a trend of an X-linked effect. Further, while we demonstrated a generic reduction in female fitness overall, an important next step would have been to demonstrate that the ML-X has negative fitness effects on females when isolated.

Reduced genetic variance revealed by inbreeding depression?

A hemiclinal analysis of variance conducted in one replicate pair of populations demonstrated a substantial reduction in genetic variance in response to selection. However, this breeding design included a potential artefact in the form of a Y chromosome local to selection conditions, complicating the interpretation of a reduction in variance in response to alleviation of SA balancing selection. In congruence with our assays of male fitness in the presence of the artefactual Y, selected haplotypes displayed a significant fitness advantage over controls. I found no changes in heritable variation underlying female fecundity, or the intersex genetic correlation for these traits between the two treatments.

Our analysis of fitness revealed that ML_{DD} (ML-double dose) males had significantly lower fitness than both ML_{SD} and control males. This could be due to costs associated with the exclusively haploid mode of selection employed. For instance, if ML selection resulted in an overexpression of certain alleles to compensate for deleterious variation present in the genetic background. While beneficial in a haploid state, this could result in fitness costs in a diploid state. Alternatively though, this could point to inbreeding depression – as a consequence of reduced genetic variation through ML selection.

Harmless males

An extremely curious result from the selection experiment was the evolution of reduced male mate harm. Previously, evolved reductions in harm have only been identified under conditions of monogamy (or female biased sex ratios) where male and female reproductive outcomes are equalized, or in response to energetic trade-offs. Here, we wonder if the extreme susceptibility of clone generator (CG) females accidentally harmonizes their reproductive outcomes with male mates. For instance, if the only females that survived the two days of mating to get to oviposition on day 14 are those mated with the least-harming males, said males monopolize the reproductive output of the population cage. Early

generations of the selection experiment did witness higher female death and lower fecundity (personal observation), which I (perhaps) mistakenly understood as improved animal husbandry skills on our part.

Paved road, no driver

X-linked meiotic drive is typically discovered in the context of obvious deviations from Fisherian sex ratios. As a consequence, known driving sex chromosomes have usually proliferated through a population, or have been discovered after through the evolution of drive resistance. In this experiment, the exclusive evolutionary success of the ML-X chromosome (Ys always hit a dead-end) provides the perfect conditions for selection to act on genetic variance coding for meiotic drive, and reveal early nascent drive trajectory. In spite of this, we found no deviation from Fisherian sex ratios in our assays, suggesting an absence of driving elements in the standing genetic variance.

Forget me dot

Besides assaying fitness and locomotion, Abbott *et al.* (2020) also performed a transcriptome analysis to study expression changes associated with ML-X selection. Surprisingly, they found that largest change in up-regulation of expression (relative to neutral expectations) is on the 4th chromosome. As a “dot” chromosome with < 1% of the genes in the genome, the 4th chromosome has consistently been ignored entirely in both male-limited selection experiments and hemiclinal analysis. Abbott *et al.* challenge the assumption that the size of the dot chromosome implies a lack of function.

Recently, Geeta Arun (2022) proposed that the dot chromosome may actually be a pertinent region of the genome to study for the accumulation of sexually antagonistic variance. As a post-X chromosome (ancestrally an X, currently an autosome), the 4th chromosome has an evolutionary history that may have eased the conditions of invasion for SA alleles. However, it is no longer inherited asymmetrically, allowing for accurate

quantitative genetic estimations of the quantum of SA variation segregating upon it. Lastly, the 4th chromosome is non-recombining, which simplifies the logistics of producing iso-4 lines, and introgressing them into a variety of genetic backgrounds to study sexually antagonistic fitness effects.

You get what you select for: Experimental caution

Overall, this thesis is a testament to the need for caution in experimental design and selection of base populations. One of the central findings of the ML selection experiment is that the animals adapted to cytogenetic constructs utilized to enable sex-limited selection, obscuring our ability to draw clear conclusions about (1) sexual antagonism in the standing genetic variance of populations examined, (2) changes in genetic variance in response to selection, and (3) the genomic distribution of SA fitness effects.

In the process of conducting these experiments, I became extremely concerned with a second category of experimental oversight. Around 30 generations into the selection experiment, I conducted a first round of male fitness assays. The breeding design used to achieve a truly generic test of fitness was unnecessarily complicated, and the breakdown of a marker associated with translocated autosomes led us to suspect that our finding (of no differences between selection treatments, even back then) was just an error. Come generation 50 however, a second round of experimentation with a much tighter protocol reproduced our initial results. In our haste to pivot projects, had we started a sex-limited selection project in a population of fruit flies with no SA variation? On its own, this did not represent a concern. An absence of SA variation in a novel base population was a finding worth demonstrating, especially given that nearly all the IaSC research in *D. melanogaster* had been conducted on a single source population (discussed in chapter 2), limiting the generality of findings. That said, demonstrating that a population with no genetic variance whatsoever also had no SA variance would have been a spectacular waste of time. Although outbred and maintained at

large census sizes, the base population employed in this selection experiment had a long history of laboratory domestication. Decades of consistent selection pressures may have acted to fix alleles, even at loci undergoing with balancing selection given a small net directional selection coefficient.

With this concern in mind, I worked with Troy Day to model trait covariance trajectories under laboratory domestication. Given my interest in SA fitness effects, I wanted to test if the covariance between male and female fitness presented a predictable trajectory, and if so – whether we could make any educated guesses about the nature of variation in our long domesticated population. In a debate over life-history trade-offs, Service & Rose (1985) had offered a verbal model to explain trait covariance trajectories during domestication. In general, novel environments are known to destabilize fitness functions acting on populations and artificially positivize trait covariances between fitness components that may otherwise trade-off against one another. Further, the lab represents a very specific case of a novel environment, frequently presenting a very gentle conditions in terms of the natural stressors presented to these animals. Absent predation, resource limitation, seasonal cycles (which may or may not be longer than the organism's life cycle) – plenty of the variation in the newly introduced population does not influence fitness the way it does in the wild. Service & Rose argued that a fitness class of fortuitously pre-adapted animals should be selected towards fixation, gradually exhausting positive genetic correlations. At or near selective equilibrium, negative genetic covariance between fitness components should dominate – potentially re-illuminating previously obscured trade-offs.

To study this, we repeatedly sampled estimates for a pair of fitness components and explicitly calculated the change in trait covariance over generations of stable “laboratory” maintenance, across a parameter space that included a variety of trait distributions, sampling systems and fitness functions. Covariance trajectories were qualitatively similar across the

parameter space tested. Broadly, our calculations echoed the predictions of Service & Rose. We were able to meaningfully separate the timeline into three phases – early, middle, and late-stage domestication. The stereotypical picture that emerged was that of a population that initially displayed positive covariances in the early phase, before turning negative in the middle and finally approaching zero in the late stages. Using a diagnostic calculation, we showed that more than half the iterations followed this trajectory. While not all iterations demonstrated positive covariances in the early stage, positive covariances were exclusively seen in this phase. Negative covariances were seen in both early and middle phases, but the magnitude of negative covariance was always greatest in the middle. All iterations with no exceptions ended with no heritable variation, resulting in covariance estimates of zero.

The model we developed was largely a simple toy – it considers only additive genetic variance, allows for no mutations etc. Nevertheless, we found clear parallels to these patterns in the trade-off literature. Life history trait measurements in populations freshly introduced to the laboratory / common garden, on fruit flies (Murphy *et al.* 1983, Klepsatel *et al.* 2013), plants (Siemens & Mitchell-Olds 1998), freshwater invertebrates (Bell *et al.* 1984a, 1984b), and mosquitofish (Stearns 1983) consistently found positive correlations between traits canonically expected to undergo trade-offs (Stearns 1989). By contrast, evidence for the existence of life-history trade-offs in fitness components is derived entirely from evolutionarily equilibrated populations displaying negative correlations between components of fitness like longevity and early age fecundity (Rose & Charlesworth 1981, Rose 1984, Luckinbill *et al.* 1984, Zwaan *et al.* 1995b) or stress tolerance and mass-specific metabolic rate (Hoffmann & Parsons 1989, Harshman & Hoffmann 1999) (reviewed in Prasad & Joshi 2003).

The same pattern was readily visible with IaSC as well. Experimental work on freshly domesticated animals reveals positive intersex genetic covariances for fitness - in crickets

(Zajitschek *et al.* 2007), stickleback fish (Kim *et al.* 2016), and fruit flies (Nguyen *et al.* 2017); whereas evidence for negative intersex covariances for fitness is consistently from evolutionarily equilibrated populations - in fruit flies (Chippindale *et al.* 2001), red flour beetles (Pai & Yan 2002), ground crickets (Fedorka & Mousseau 2004), moths (Lewis *et al.* 2011), and seed beetles (Berger *et al.* 2014). Although tangential to our original question about late stage populations, these findings provide a useful guideline to experimentalists interested in assaying IaSC in populations. Newly domesticated populations require a few generations to equilibrate to conditions. This process undoubtedly changes a population, through the selective elimination of “lab unfit” variation, making for poor replicas of wild populations. Nevertheless, this is still more informative of the genetic architecture of populations relative to snapshots immediately after a transplant from the field to the lab.

A common alternative is the use of iso-female sampling. The rationale behind the creation of isofemale lines is the preservation of greater natural variation, as against the loss of variants experienced due to lab equilibration. However, due to the elevated relatedness of animals breeding within isofemale lines, they are likely to reveal (and potentially eliminate) recessive deleterious variation. Additionally, when synthetic populations are reconstituted from isofemale lines, the resulting variation must undergo early laboratory selection anyway – presenting these populations with two steps of costs rather than one.

Returning to our original concern of late-stage populations however, our model consistently demonstrated that populations under long term homogenous selection completely lose genetic variance. Contrary to these expectations, our selection experiment demonstrated substantial adaptive responses. Besides evolved changes in fitness under various test conditions, I also found evidence for partial masculinization of the phenotype, through measurements of body size and development time (not described in thesis). ML animals of both sexes developed slower and grew larger than controls, demonstrating positive genetic

correlations for both traits. While selection shifted development time towards the male direction of extant dimorphism in the species, it moved body size in the opposite direction. It must be noted that increased body size has been found to be beneficial to the sexual fitness of males in a number of studies on *Drosophila*. Our results may point at a need to rethink our simplistic understanding of masculinization as a movement towards the male direction of dimorphism. In any case, while our model is able to predict the trajectory of trait covariances in early and middle phases of laboratory domestication, it does not accurately capture the nature of variation in late stage populations, necessitating more nuanced theorization about the genetic architecture of balanced polymorphisms.

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APPENDIX A

Supplementary Material

Intralocus sexual conflict explored through male-limited selection in *Drosophila melanogaster*: A failure to replicate.

Table A1a. The results of the ANOVA fit for the male CRF - generations 50, 64

<i>Response</i>		<i>Variable</i>	<i>df</i>	<i>F</i>	<i>P</i>
Fitness	Gen 50	Selection	1	0.9711	0.33
		Replicate	2	2.2390	0.12
		Sel:Rep	2	1.2323	0.30
Fitness	Gen 64	Selection	1	0.5599	0.46
		Replicate	2	0.7995	0.46
		Sel:Rep	2	0.7617	0.48

Table A1b. The results of the ANOVA fit for the male CRF - generation 70

<i>Response</i>		<i>Variable</i>	<i>df</i>	<i>F</i>	<i>P</i>
Fitness	Gen 70	Treatment	1	7.3354	0.0003
		Replicate	2	1.6979	0.19
		Sel:Rep	2	2.2094	0.0543

Table A1c. Contrast testing between levels of treatment in male CRF (gen 70) ANOVA. P value adjustment: Tukey method for comparing a family of 4 estimates

<i>Contrast</i>	<i>Estimate</i>	<i>SE</i>	<i>t ratio</i>	<i>df</i>	<i>P</i>
MC:MLDD	0.0789	0.0244	3.237	68	0.0099
MC:MLSD	-0.0221	0.0244	-0.907	68	0.80
MC:MLSDa	0.0336	0.0244	1.378	68	0.52
MLDD:MLSD	-0.1010	0.0244	-4.144	68	0.0006
MLDD:MLSDa	-0.0453	0.0244	-1.858	68	0.26
MLSD:MLSDa	0.0557	0.0244	2.285	68	0.11

Table A2a. The results of the F test for male CRF heritable variance

<i>Response</i>	<i>Variable</i>		<i>MS</i>	<i>df</i>	<i>F</i>	<i>P</i>
Fitness	Line	MC	0.17705	37	3.4798	~0.0001
Fitness	Line	ML	0.05088	37		

Table A2b. The results of the F test for female productivity heritable variance

<i>Response</i>	<i>Variable</i>		<i>MS</i>	<i>df</i>	<i>F</i>	<i>P</i>
Fitness	Line	ML	0.4670	37	1.1013	0.38
Fitness	Line	MC	0.4240	37		

Table A2c. The results of ANOVAs on male CRF and female productivity modelled by selection.

<i>Response</i>	<i>Variable</i>	<i>df</i>	<i>F</i>	<i>P</i>
Male CRF	Selection	1	17.766	<0.0001
Female productivity	Selection	1	1.1464	0.29

Table A2d. The results of intersex genetic correlation (r_{mf}) tests on male CRF and female productivity.

	<i>Estimate</i>	<i>df</i>	<i>t</i>	<i>P</i>
ML	0.0247	36	0.1481	0.88
MC	0.1062	36	0.6408	0.53

Table A2e. The results of a two-tailed Fisher's Z test comparing (r_{mf}) estimates from ML and MC lines

	<i>Estimate</i>	<i>z</i>	<i>P</i>
MC-ML	0.0815	0.4195	0.34

Table A3a. The results of the ANOVA fit for the female CRF - generations 48, 50

<i>Response</i>		<i>Variable</i>	<i>df</i>	<i>F</i>	<i>P</i>
Fitness	Gen 48	Selection	1	43.928	<0.0001
		Replicate	2	2.0172	0.15
		Sel:Rep	2	1.5259	0.23
Fitness	Gen 50	Selection	1	91.803	<0.0001
		Replicate	2	14.285	<0.0001
		Sel:Rep	2	12.637	~0.0001

Table A3b. Pairwise contrast testing between selection treatments within each level of replicate. Adjusted alpha rate for 3 comparisons is $\alpha = 0.0169$.

<i>Response</i>	<i>Replicate</i>	<i>Variable</i>	<i>df</i>	<i>F</i>	<i>P</i>
Fitness	1	Selection	1	41.830	<0.0001
	3	Selection	1	68.547	<0.0001
	5	Selection	1	3.9771	0.0741

Table A4a. The results of the ANOVA fit on a generalized linear model (binomial error distribution) for male mating success.

<i>Response</i>	<i>Variable</i>	<i>df</i>	<i>F</i>	<i>P</i>
Mating Success	Selection	1	2.0752	0.15
	Replicate	2	1.7469	0.18
	Sel:Rep	2	0.1189	0.88

Table A4b. The results of the ANOVA fit for target male mating latency.

<i>Response</i>	<i>Variable</i>	<i>df</i>	<i>F</i>	<i>P</i>
Mating Latency	Selection	1	1.4846	0.22
	Replicate	2	0.7299	0.48
	Sel:Rep	2	1.1045	0.34

Table A4c. The results of the ANOVA fit for target male mating duration.

<i>Response</i>	<i>Variable</i>	<i>df</i>	<i>F</i>	<i>P</i>
Mating Duration	Selection	1	0.2969	0.59
	Replicate	2	1.1592	0.32
	Sel:Rep	2	0.2941	0.75

Table A4d. The results of the ANOVA fit for fecundity induced by target males.

<i>Response</i>	<i>Variable</i>	<i>df</i>	<i>F</i>	<i>P</i>
Fecundity induced	Selection	1	0.1000	0.75
	Replicate	2	1.0035	0.37
	Sel:Rep	2	0.3706	0.69

Table A4e. The results of the ANOVA fit for brood sex ratio of target male's offspring.

<i>Response</i>	<i>Variable</i>	<i>df</i>	<i>F</i>	<i>P</i>
Sex ratio	Selection	1	0.0609	0.81
	Replicate	2	1.7600	0.18
	Sel:Rep	2	2.6786	0.0726

Table A5a. The results of the ANOVA fit on a generalized linear model (binomial error distribution) on the number of target males that sired 100% of their mate's offspring.

<i>Response</i>	<i>Variable</i>	<i>df</i>	<i>F</i>	<i>P</i>
WT only Progeny	Treatment	1	1.5359	0.20
	Replicate	2	0.2647	0.77
	Sel:Rep	2	1.1248	0.35

Table A5b. The results of the ANOVA fit for offspring sired, by target males that did not sire 100% of their mate's offspring.

<i>Response</i>	<i>Variable</i>	<i>df</i>	<i>F</i>	<i>P</i>
P2	Treatment	1	1.6598	0.20
	Replicate	2	2.2473	0.11
	Sel:Rep	2	1.3440	0.26

Figure A1. (a) ML selection breeding design. (b) Recombination box design. CG females are denoted as DTP or DTW based on presence or absence of bw^D marker respectively.

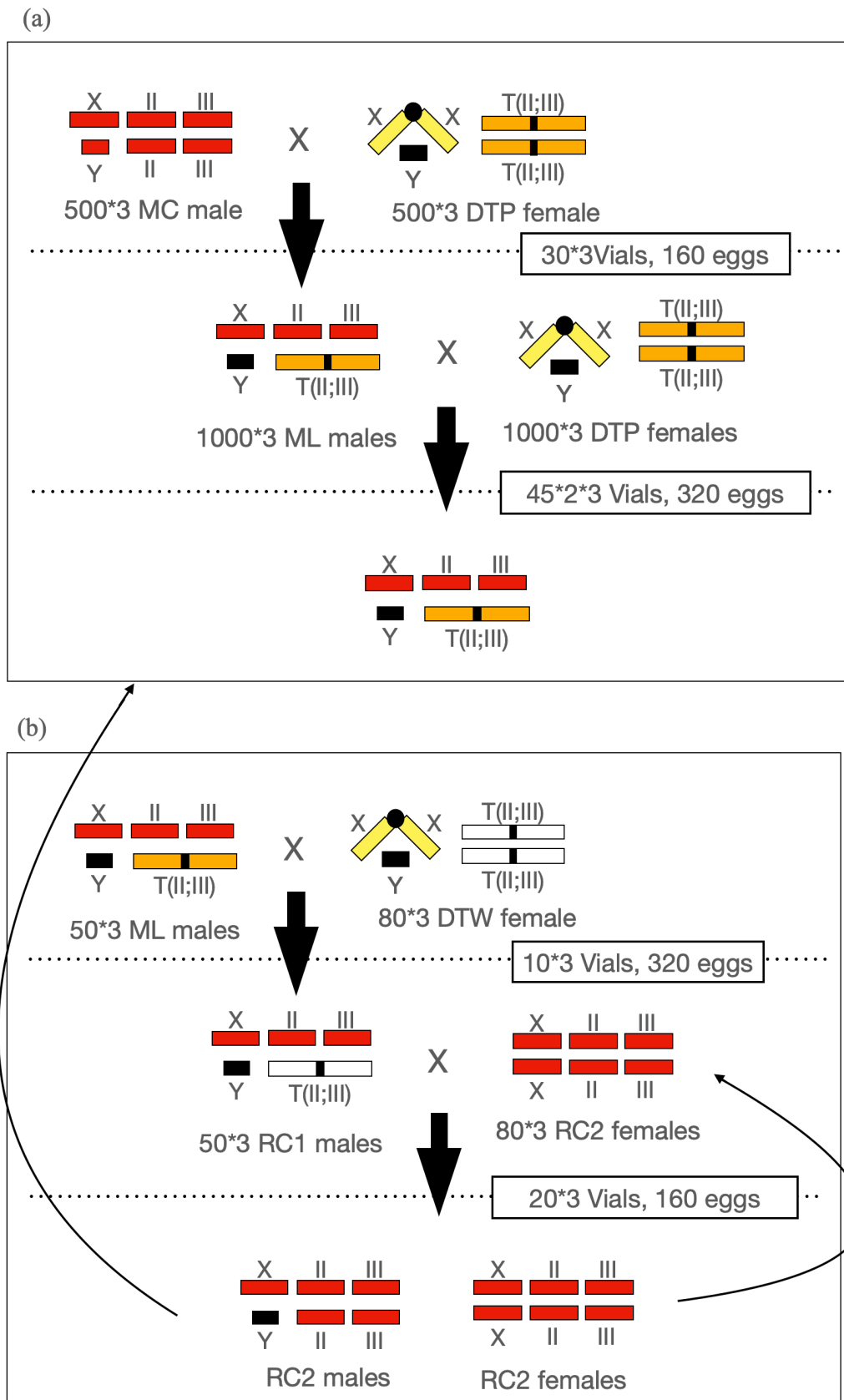
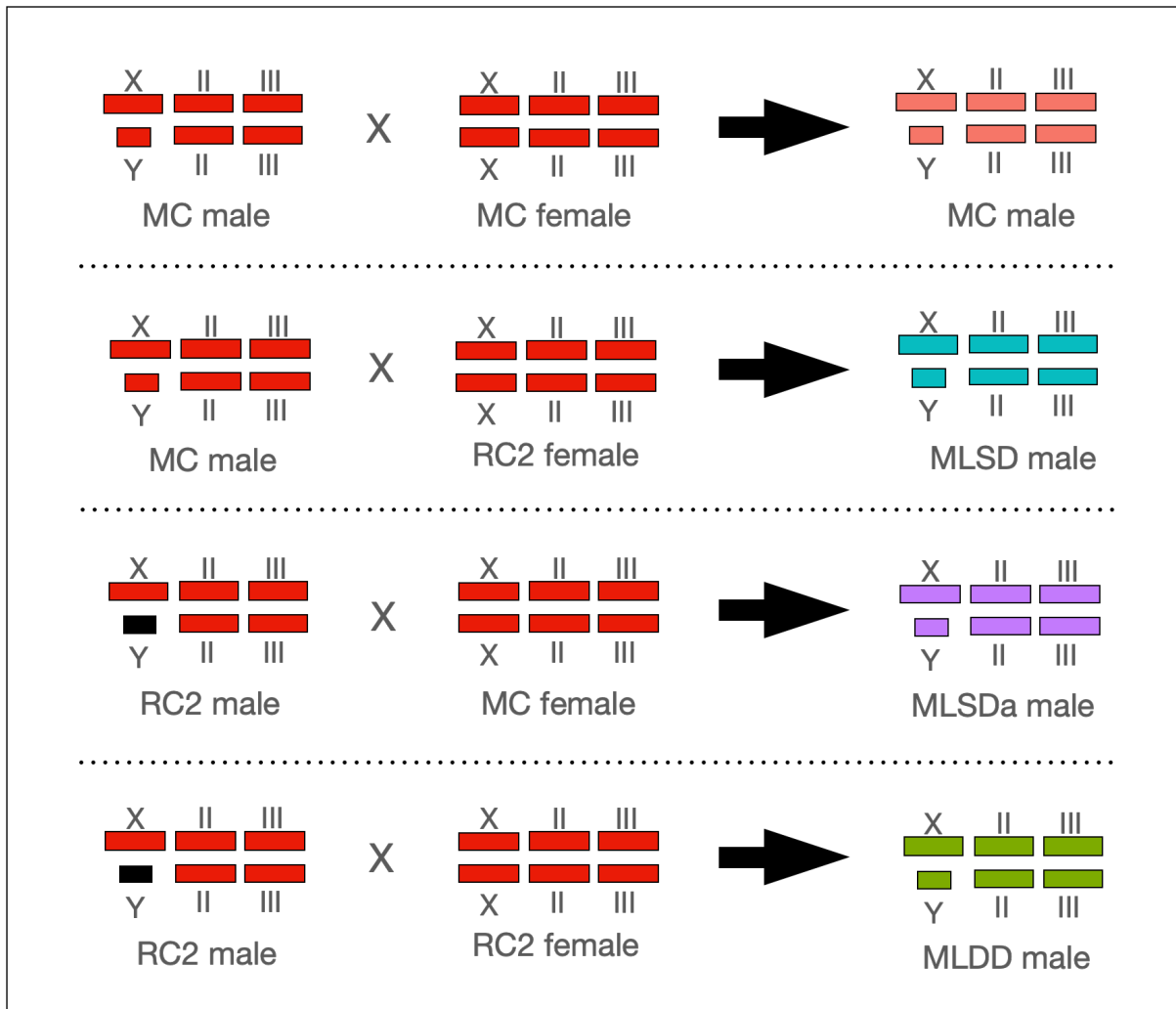


Figure A2. CRF experimental males:

(a)



(b)

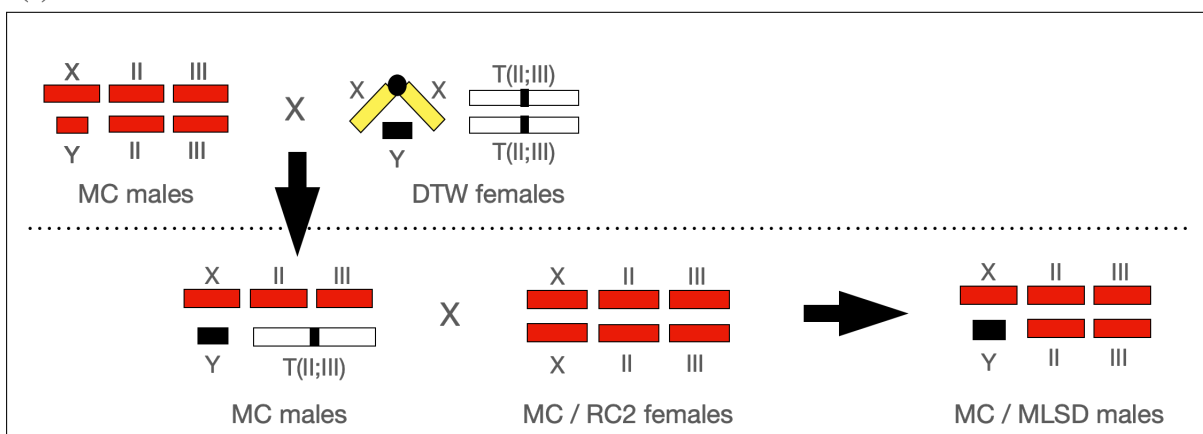
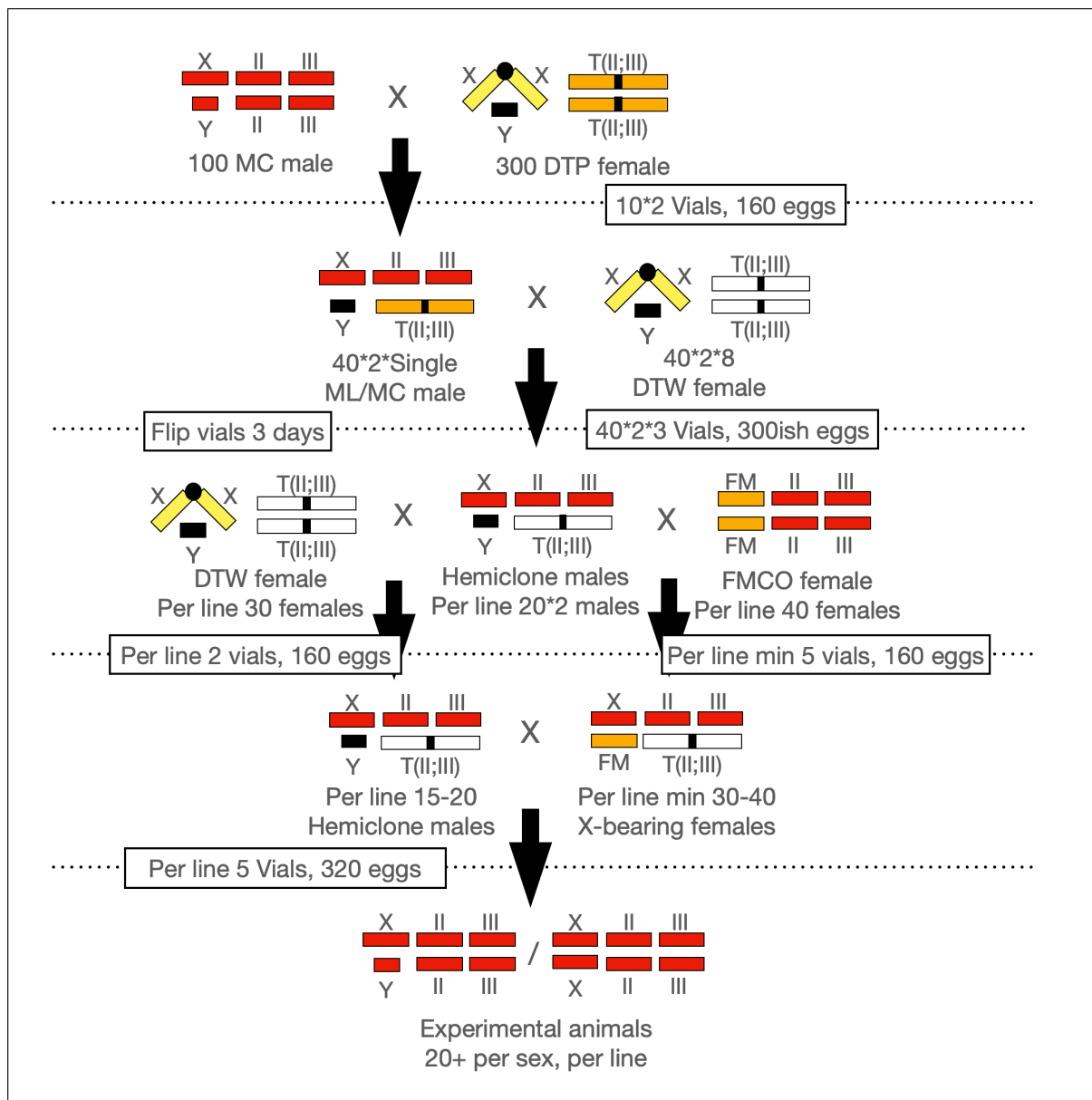


Figure A3. Hemiclonal analysis – breeding design



APPENDIX B

Supplementary Material

Compensatory evolution dominates response in a sex-limited selection experiment in *Drosophila melanogaster*.

Table B1. The results of the fully factorial ANOVA fit for the male CRF.

<i>Response</i>	<i>Variable</i>	<i>df</i>	<i>F</i>	<i>P</i>
Fitness	Selection	1	39.0051	<0.0001
	Replicate	2	0.4380	0.65
	Female	1	0.7970	0.37
	Bg	1	182.9934	<0.0001
	Sel:Rep	2	0.2625	0.77
	Sel:Fem	1	1.4094	0.24
	Sel:Bg	1	4.3954	0.0376
	Fem:Bg	1	32.1444	<0.0001
	Fem:Rep	2	1.9557	0.14
	Bg:Rep	2	0.0473	0.95
	Sel:Fem:Bg	1	1.4517	0.23
	Sel:Bg:Rep	2	0.9330	0.39
	Sel:Fem:Rep	2	0.0503	0.95
	Fem:Bg:Rep	2	0.1795	0.84
	Sel:Fem:Bg:Rep	2	2.4533	0.0894

Table B1a. The results of the ANOVA fits for the male CRF in each combination of female and background. Adjusted alpha rate for 4 comparisons is $\alpha = 0.01274$

<i>Response</i>	<i>Female</i>	<i>Background</i>	<i>Variable</i>	<i>df</i>	<i>F</i>	<i>P</i>
Fitness	CG	HC	Selection	1	29.4353	<0.0001
			Replicate	2	1.5767	0.22
			Sel:Rep	2	3.0820	0.0542
Fitness	Cr	HC	Selection	1	59.8226	<0.0001
			Replicate	2	3.1641	0.0567
			Sel:Rep	2	0.1099	0.90
Fitness	CG	WT	Selection	1	5.8642	0.0189
			Replicate	2	0.0822	0.92
			Sel:Rep	2	0.6021	0.55
Fitness	Cr	WT	Selection	1	0.5599	0.46
			Replicate	2	0.7995	0.46
			Sel:Rep	2	0.7617	0.48

Table B2a. The results of the fully factorial ANOVA fit for the male mating success.

<i>Response</i>	<i>Variable</i>	<i>df</i>	<i>F</i>	<i>P</i>
Mating Success	Selection	1	11.852	0.0006
	Replicate	2	6.9319	0.0010
	Female	1	6.0212	0.0144
	Background	1	1.8860	0.17
	Sel:Rep	2	0.2892	0.75
	Sel:Fem	1	0.2098	0.65
	Sel:Bg	1	0.0339	0.85
	Fem:Bg	1	0.0978	0.75
	Fem:Rep	2	0.0025	0.99
	Bg:Rep	2	0.6487	0.52
	Sel:Fem:Bg	1	0.4053	0.52
	Sel:Bg:Rep	2	1.2380	0.29
	Sel:Fem:Rep	2	2.5206	0.081
	Fem:Bg:Rep	2	0.7242	0.48
	Sel:Fem:Bg:Rep	2	0.4756	0.62

Table B2b. The results of the ANOVA fits for the male mating success in each replicate. Adjusted alpha rate for 3 comparisons is $\alpha = 0.0169$

<i>Response</i>	<i>Replicate</i>	<i>Variable</i>	<i>df</i>	<i>F</i>	<i>P</i>
Mating Success	1	Selection	1	5.7934	0.0166
		Background	1	0.0895	0.76
		Female	1	2.0137	0.16
		Sel:Bg	1	1.3593	0.24
		Sel:Fem	1	2.9241	0.0881
		Bg:Fem	1	0.0427	0.84
		Sel:Bg:Fem	1	0.4183	0.52
Mating Success	3	Selection	1	4.6478	0.0318
		Background	1	2.9265	0.0880
		Female	1	2.0929	0.15
		Sel:Bg	1	0.2155	0.64
		Sel:Fem	1	0.3033	0.58
		Bg:Fem	1	1.3429	0.25
		Sel:Bg:Fem	1	0.7699	0.38
Mating Success	5	Selection	1	1.9735	0.16
		Background	1	0.1392	0.71
		Female	1	1.9094	0.17
		Sel:Bg	1	1.0175	0.31
		Sel:Fem	1	2.0905	0.15
		Bg:Fem	1	0.1635	0.69
		Sel:Bg:Fem	1	0.1683	0.68

Table B2c. The results of the fully factorial ANOVA fit for the male mating latency.

<i>Response</i>	<i>Variable</i>	<i>df</i>	<i>F</i>	<i>P</i>
log(latency+1)	Selection	1	4.8707	0.0277
	Replicate	2	2.4328	0.0887
	Female	1	0.0036	0.48
	Background	1	0.5040	0.95
	Sel:Rep	2	2.6575	0.0710
	Sel:Fem	1	0.3154	0.57
	Sel:Bg	1	0.1183	0.73
	Fem:Bg	1	2.4795	0.12
	Fem:Rep	2	1.7631	0.17
	Bg:Rep	2	0.2983	0.74
	Sel:Fem:Bg	1	0.5340	0.46
	Sel:Bg:Rep	2	0.1233	0.88
	Sel:Fem:Rep	2	2.0226	0.13
	Fem:Bg:Rep	2	0.2421	0.78
	Sel:Fem:Bg:Rep	2	2.0371	0.13

Table B2d. The results of the fully factorial ANOVA fit for the male mating duration.

<i>Response</i>	<i>Variable</i>	<i>df</i>	<i>F</i>	<i>P</i>
Duration	Selection	1	5.7565	0.0168
	Replicate	2	3.9574	0.0197
	Female	1	13.122	0.0003
	Background	1	11.632	0.0007
	Sel:Rep	2	0.4611	0.63
	Sel:Fem	1	0.8901	0.34
	Sel:Bg	1	2.9102	0.0885
	Fem:Bg	1	0.0755	0.78
	Fem:Rep	2	0.0931	0.91
	Bg:Rep	2	0.3389	0.71
	Sel:Fem:Bg	1	1.0318	0.31
	Sel:Bg:Rep	2	0.1324	0.88
	Sel:Fem:Rep	2	0.0675	0.93
	Fem:Bg:Rep	2	0.2644	0.77
	Sel:Fem:Bg:Rep	2	1.4654	0.23

Table B2e. The results of the ANOVA fits for the male mating duration in each replicate. Adjusted alpha rate for 3 comparisons is $\alpha = 0.0169$

<i>Response</i>	<i>Replicate</i>	<i>Variable</i>	<i>df</i>	<i>F</i>	<i>P</i>
Mating Duration	1	Selection	1	0.6031	0.44
		Background	1	6.2255	0.0134
		Female	1	7.2311	0.0077
		Sel:Bg	1	2.4218	0.12
		Sel:Fem	1	0.0859	0.77
		Bg:Fem	1	0.5709	0.45
		Sel:Bg:Fem	1	0.0740	0.78
Mating Duration	3	Selection	1	2.8546	0.0928
		Background	1	1.4336	0.23
		Female	1	3.3933	0.0671
		Sel:Bg	1	0.3654	0.55
		Sel:Fem	1	0.6404	0.42
		Bg:Fem	1	0.1182	0.72
		Sel:Bg:Fem	1	0.0285	0.86
Mating Duration	5	Selection	1	2.9956	0.085
		Background	1	5.4032	0.0214
		Female	1	3.0061	0.085
		Sel:Bg	1	0.7247	0.39
		Sel:Fem	1	0.1817	0.67
		Bg:Fem	1	0.0225	0.88
		Sel:Bg:Fem	1	3.5357	0.062

Table B2f. The results of the fully factorial ANOVA fit for the fecundity induced by males.

<i>Response</i>	<i>Variable</i>	<i>df</i>	<i>F</i>	<i>P</i>
Fecundity induced	Selection	1	0.3654	0.55
	Replicate	2	4.3529	0.0133
	Female	1	782.20	<0.0001
	Background	1	148.61	<0.0001
	Sel:Rep	2	0.4006	0.67
	Sel:Fem	1	0.1327	0.72
	Sel:Bg	1	2.5480	0.11
	Fem:Bg	1	21.246	<0.0001
	Fem:Rep	2	1.2105	0.30
	Bg:Rep	2	0.3138	0.73
	Sel:Fem:Bg	1	0.0742	0.78
	Sel:Bg:Rep	2	1.4759	0.23
	Sel:Fem:Rep	2	0.0181	0.98
	Fem:Bg:Rep	2	0.1891	0.83
	Sel:Fem:Bg:Rep	2	1.7209	0.18

Table B2g. The results of the ANOVA fits for the fecundity induced by target males in each combination of female and background. Adjusted alpha rate for 4 comparisons is $\alpha = 0.01274$

<i>Response</i>	<i>Female</i>	<i>Background</i>	<i>Variable</i>	<i>df</i>	<i>F</i>	<i>P</i>
Fecundity induced	CG	HC	Selection	1	3.3938	0.0678
			Replicate	2	3.1436	0.0465
			Sel:Rep	2	0.2288	0.79
Fecundity induced	Cr	HC	Selection	1	2.0226	0.16
			Replicate	2	2.3028	0.10
			Sel:Rep	2	2.6918	0.0719
Fecundity induced	CG	WT	Selection	1	0.0000	0.99
			Replicate	2	0.8594	0.42
			Sel:Rep	2	0.2003	0.82
Fecundity induced	Cr	WT	Selection	1	0.1394	0.71
			Replicate	2	0.8620	0.42
			Sel:Rep	2	0.4580	0.63

Table B2h. The results of the fully factorial ANOVA fit for the sex ratio of broods sired by target males.

<i>Response</i>	<i>Variable</i>	<i>df</i>	<i>F</i>	<i>P</i>
Brood sex ratio	Selection	1	2.2747	0.15
	Replicate	2	0.1619	0.85
	Female	1	0.0126	0.91
	Background	1	0.3660	0.55
	Sel:Rep	2	0.4732	0.62
	Sel:Fem	1	1.1934	0.27
	Sel:Bg	1	0.3567	0.55
	Fem:Bg	1	0.0863	0.77
	Fem:Rep	2	0.8309	0.44
	Bg:Rep	2	1.3983	0.25
	Sel:Fem:Bg	1	0.1977	0.66
	Sel:Bg:Rep	2	0.7795	0.46
	Sel:Fem:Rep	2	1.0350	0.36
	Fem:Bg:Rep	2	0.0947	0.90
	Sel:Fem:Bg:Rep	2	0.7588	0.47

Table B3a. The results of the fully factorial ANOVA fit on a generalized linear model (binomial error distribution) on the number of target males that sired 100% of their mate's offspring.

<i>Response</i>	<i>Variable</i>	<i>df</i>	<i>F</i>	<i>P</i>
P2 part 1	Selection	1	0.0158	0.90
	Replicate	2	0.3192	0.73
	Female	1	3.9988	0.0458
	Background	1	44.164	<0.0001
	Sel:Rep	2	0.9428	0.39
	Sel:Fem	1	1.3406	0.25
	Sel:Bg	1	0.0784	0.78
	Fem:Bg	1	27.517	<0.0001
	Fem:Rep	2	1.7691	0.17
	Bg:Rep	2	1.4932	0.22
	Sel:Fem:Bg	1	0.1202	0.73
	Sel:Bg:Rep	2	2.2303	0.11
	Sel:Fem:Rep	2	1.9455	0.14
	Fem:Bg:Rep	2	0.2614	0.77
	Sel:Fem:Bg:Rep	2	0.9112	0.40

Table B3b. The results of the fully factorial ANOVA fit for offspring sired, by target males that did not sire 100% of their mate's offspring.

<i>Response</i>	<i>Variable</i>	<i>df</i>	<i>F</i>	<i>P</i>
P2	Selection	1	0.7596	0.38
	Replicate	2	0.2658	0.77
	Female	1	86.891	<0.0001
	Background	1	138.31	<0.0001
	Sel:Rep	2	0.2050	0.81
	Sel:Fem	1	0.3101	0.58
	Sel:Bg	1	0.4304	0.51
	Fem:Bg	1	87.723	<0.0001
	Fem:Rep	2	0.6061	0.55
	Bg:Rep	2	1.0976	0.34
	Sel:Fem:Bg	1	2.3676	0.12
	Sel:Bg:Rep	2	2.5523	0.08
	Sel:Fem:Rep	2	1.5697	0.21
	Fem:Bg:Rep	2	0.0424	0.96
	Sel:Fem:Bg:Rep	2	1.2971	0.28

Table B4. The results of the ANOVA fit for mate harm by target males.

<i>Response</i>	<i>Variable</i>	<i>df</i>	<i>F</i>	<i>P</i>
Mortality	Selection	1	17.648	<0.0001
	Replicate	2	1.7037	0.19
	Background	1	18.967	<0.0001
	Sel:Rep	2	1.0799	0.34
	Sel:Bg	1	0.2510	0.62
	Bg:Rep	2	1.8731	0.16
	Sel:Bg:Rep	2	1.0710	0.35
	Productivity	Selection	1	21.924
Replicate		2	0.6079	0.55
Background		1	164.21	<0.0001
Sel:Rep		2	0.0897	0.91
Sel:Bg		1	32.504	<0.0001
Bg:Rep		2	0.5131	0.60
Sel:Bg:Rep		2	1.6544	0.20

Table B5a. The results of the ANOVA fit for the male CRF, for target animals with CG autosomes.

<i>Response</i>	<i>Variable</i>	<i>df</i>	<i>F</i>	<i>P</i>
Fitness	Selection	1	2.3793	0.13
	Replicate	2	0.5659	0.57
	Sel:Rep	2	0.4788	0.62

Table B5b. The results of the ANOVA fit for the male CRF, for target animals with a CG cytotype.

<i>Response</i>	<i>Variable</i>	<i>df</i>	<i>F</i>	<i>P</i>
Fitness	Selection	1	5.2034	0.03
	Replicate	2	0.0006	0.99
	Sel:Rep	2	2.0104	0.15

Table B5c. The results of the ANOVA fit for the male CRF, for target animals with a CG Y chromosome.

<i>Response</i>	<i>Variable</i>	<i>df</i>	<i>F</i>	<i>P</i>
Fitness	Selection	1	27.1966	<0.0001
	Replicate	2	12.5216	0.0002
	Sel:Rep	2	4.9401	0.015

Table B5d. The results of the ANOVA fit for the male CRF, for target animals with a CG Y chromosome, separated by replicate. Adjusted alpha rate for 3 comparisons is $\alpha = 0.0169$

<i>Response</i>	<i>Replicate</i>	<i>Variable</i>	<i>df</i>	<i>F</i>	<i>P</i>
Fitness	1	Selection	1	24.840	0.0011
	3	Selection	1	27.085	0.0008
	5	Selection	1	0.1523	0.71

Table B5e. The results of the ANOVA fit for the female CRF, for target animals with a CG cytotype

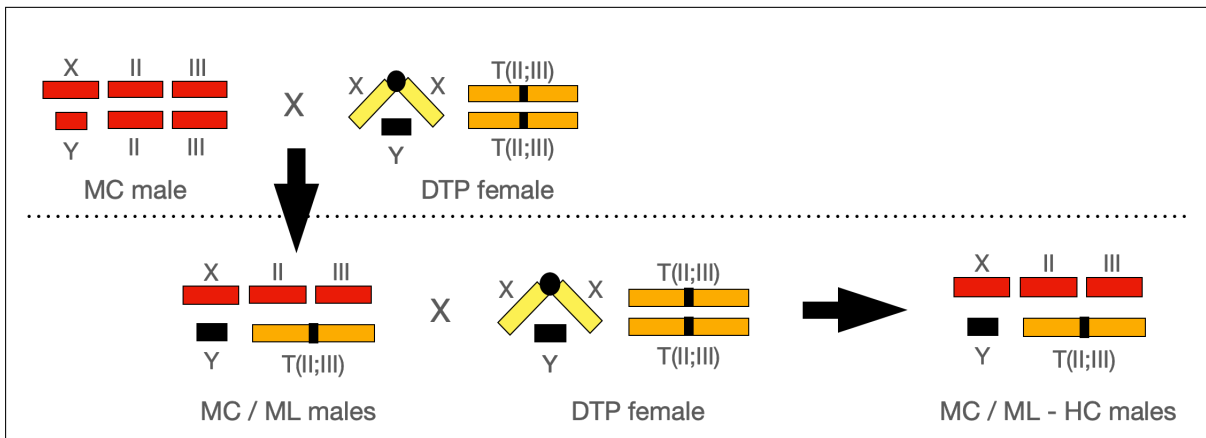
<i>Response</i>	<i>Variable</i>	<i>df</i>	<i>F</i>	<i>P</i>
Fitness	Selection	1	0.0005	0.98
	Replicate	2	8.3733	0.0003
	Sel:Rep	2	10.297	<0.0001

Table B5f. The results of the ANOVA fit for the female CRF, for target animals with a CG cytotype, separated by replicate. Adjusted alpha rate for 3 comparisons is $\alpha = 0.0169$

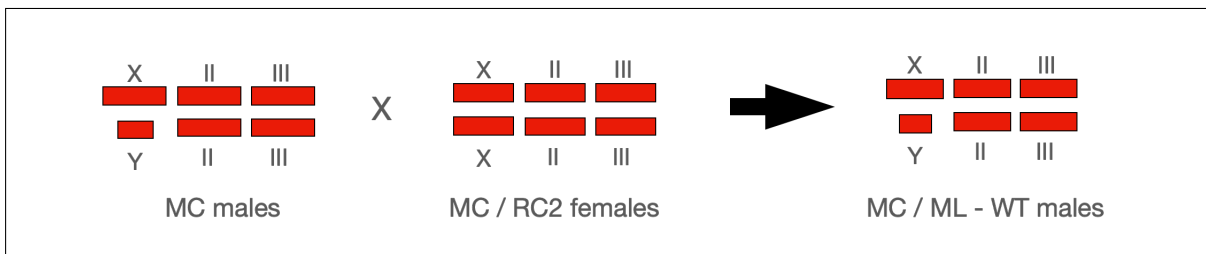
<i>Response</i>	<i>Replicate</i>	<i>Variable</i>	<i>df</i>	<i>F</i>	<i>P</i>
Fitness	1	Selection	1	1.4636	0.25
	3	Selection	1	1.2345	0.29
	5	Selection	1	8.7948	0.014

Figure B1. (a) HC males; and WT males for (b) CRF assays, and (c) mating success, sperm offense assays

(a)



(b)



(c)

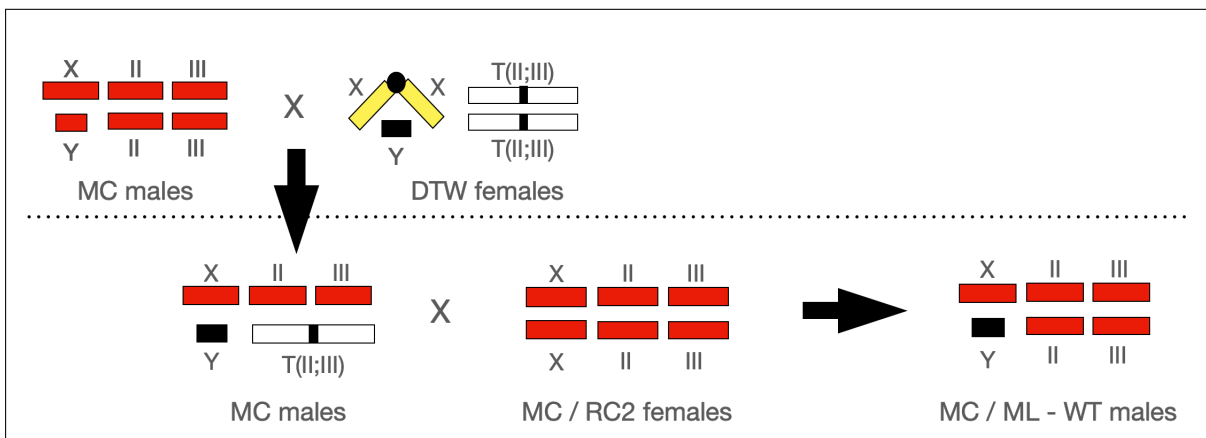


Figure B2. Single artefact males: (a) CG cyto, (b) CG Y, and (c) CG auto animals

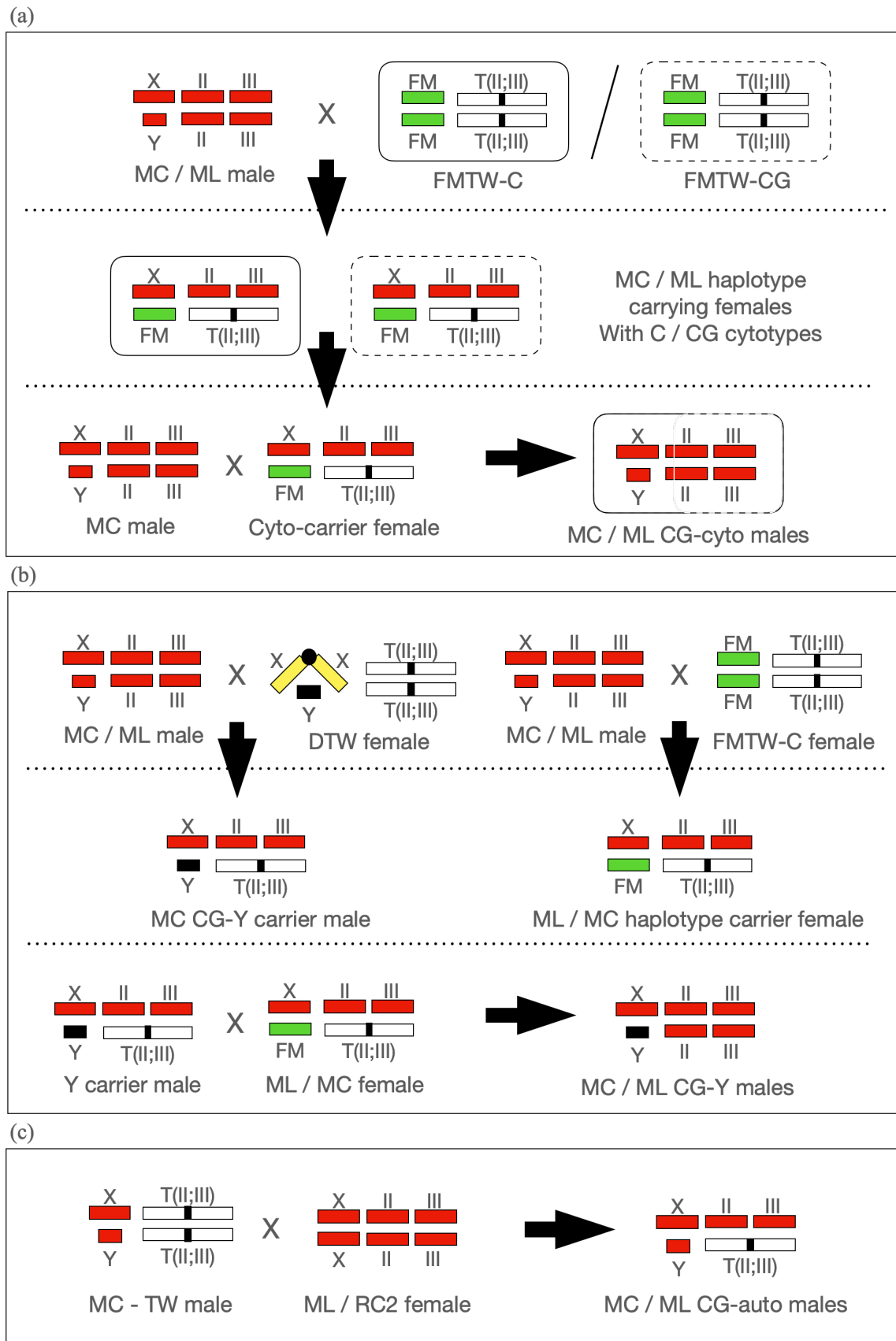
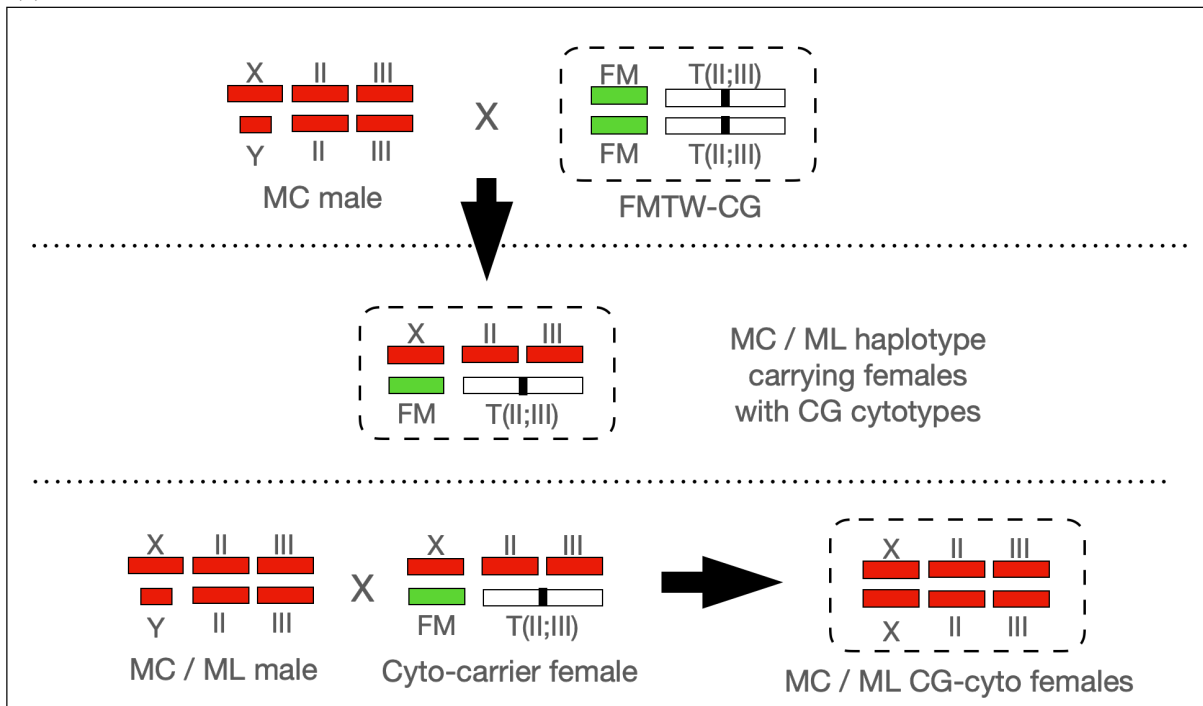
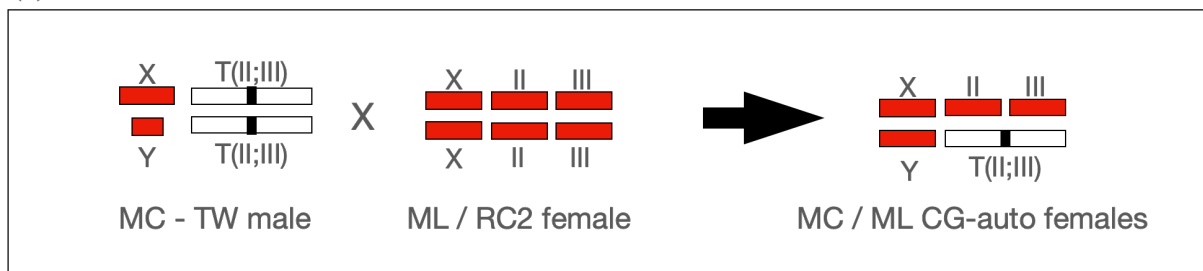


Figure B3. Single artefact females – (a) CG cyto and (b) CG auto females

(a)



(b)



APPENDIX C

Supplementary Material

Evolution of reproductive isolation in a long-term evolution experiment with *Drosophila melanogaster*: 30 years of divergent life-history selection

Figure C1. The proportion of homotypic compared to heterotypic matings recorded during the female (a), male (b), and group (c) mate choice assay for each replicate population.

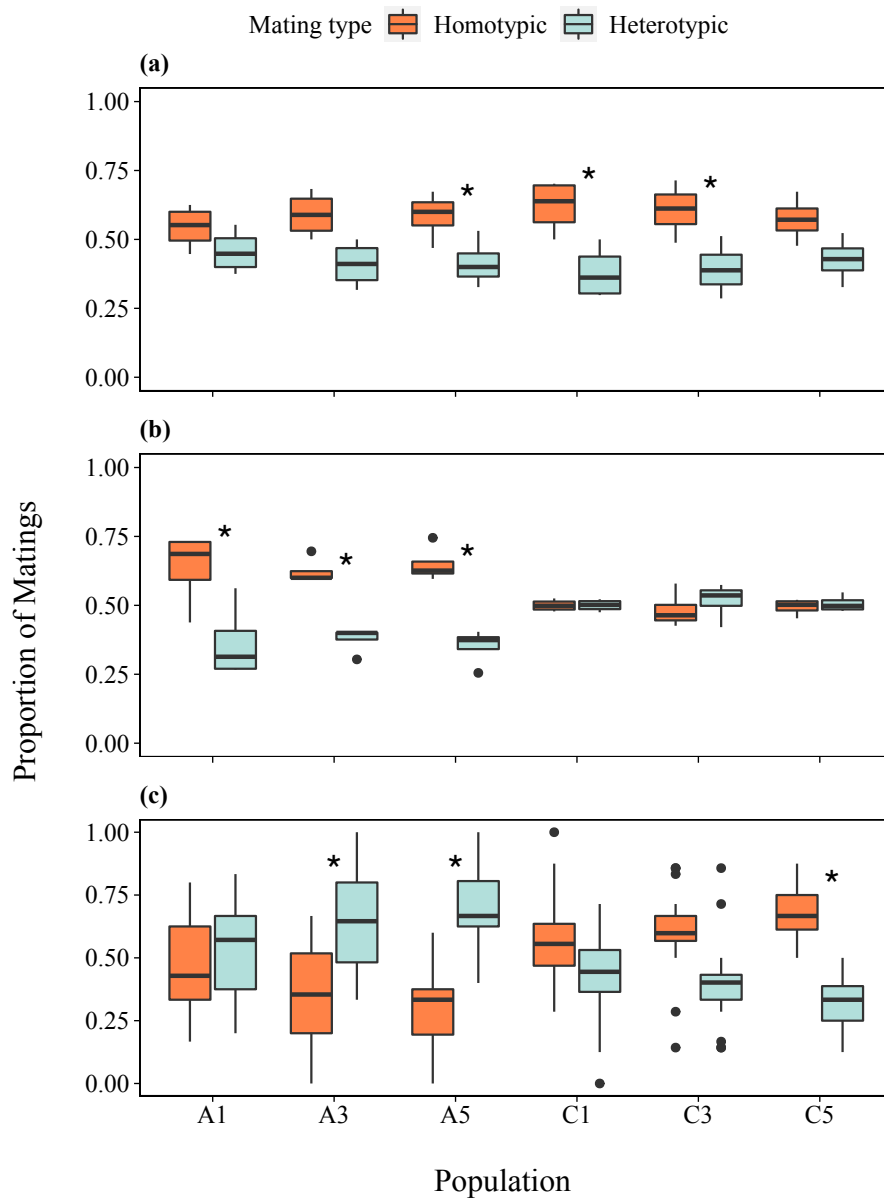


Table C1. The results of the repeated G-test for goodness of fit:

(a) Female mate choice

<i>Population</i>	<i>Het. G</i>	<i>df</i>	<i>P</i>	<i>Pooled G</i>	<i>df</i>	<i>P</i>	<i>Total G</i>	<i>df</i>	<i>P</i>
A1	3.7	3	0.3	1.55	1	0.21	5.25	4	0.26
A3	3.87	3	0.28	5.4	1	0.02	9.27	4	0.05
A5	4.54	3	0.21	5.47	1	0.02	10.02	4	0.04
Overall A	0.82	2	0.66	11.59	1	<0.001	12.42	3	0.01
C1	5.68	3	0.13	11.13	1	<0.001	16.81	4	< 0.01
C3	5.4	3	0.15	9.16	1	<0.01	14.56	4	0.01
C5	3.87	3	0.28	4.42	1	0.04	8.29	4	0.08
Overall C	0.85	2	0.65	23.86	1	<0.00001	24.71	3	< 0.0001

(b) Male mate choice

<i>Population</i>	<i>Het. G</i>	<i>df</i>	<i>P</i>	<i>Pooled G</i>	<i>df</i>	<i>P</i>	<i>Total G</i>	<i>df</i>	<i>P</i>
A1	11.62	3	0.01	13.61	1	<0.001	25.22	4	<0.0001
A3	1.42	3	0.7	11.37	1	<0.001	12.79	4	0.01
A5	3.11	3	0.38	17.85	1	<0.0001	21.11	4	<0.001
Overall A	0.41	2	0.81	46.85	1	<0.00001	47.27	3	<0.00001
C1	0.23	3	0.97	0	1	1	0.23	4	0.99
C3	2.18	3	0.54	0.29	1	0.59	2.47	4	0.65
C5	0.55	3	0.91	0.05	1	0.83	0.6	4	0.96
Overall C	0.15	2	0.93	0.19	1	0.67	0.34	3	0.95

(c) Group mate choice

<i>Population</i>	<i>Het. G</i>	<i>df</i>	<i>P</i>	<i>Pooled G</i>	<i>df</i>	<i>P</i>	<i>Total G</i>	<i>df</i>	<i>P</i>
A1	0.48	1	0.49	0.01	1	0.92	0.49	2	0.78
A3	0.58	1	0.45	7.64	1	0.01	8.23	2	0.02
A5	0.46	1	0.5	18.75	1	<0.0001	19.21	2	<0.0001
Overall A	8.87	2	0.01	17.53	1	<0.0001	26.4	3	<0.0001
C1	2.75	1	0.1	1.8	1	0.18	4.55	2	0.1
C3	0.3	1	0.58	5.13	1	0.02	5.43	2	0.07
C5	0.002	1	0.96	16.21	1	<0.0001	16.21	2	<0.001
Overall C	4.31	2	0.12	18.83	1	<0.0001	23.14	3	<0.0001

Table C2. Full LMM results for the latency and duration of matings in the female and male mate choice assays.

<i>Assay</i>	<i>Response</i>	<i>Factor Type</i>		<i>MS</i>	<i>df</i>	<i>F</i>	<i>P</i>	
Female Choice	Latency	Fixed	Male	11.36	1, 1107.2	25.71	<0.0001	
			Female	0.006	1, 1098.7	0.01	0.92	
			Male x Female	0.16	1, 1103.9	0.36	0.55	
		Random		Trial/Replicate	% var	Df	X ²	P
					4.85	2	28.77	<0.0001
					<i>MS</i>	<i>df</i>	<i>F</i>	<i>P</i>
Female Choice	Duration	Fixed	Male	0.05	1, 1105.1	0.003	0.96	
			Female	788.89	1, 1098.3	52.19	<0.0001	
			Male x Female	268.75	1, 1101.5	17.78	<0.0001	
		Random		Trial/Replicate	% var	Df	X ²	P
					4.48	2	24.91	<0.0001
					<i>MS</i>	<i>df</i>	<i>F</i>	<i>P</i>
Male Choice	Latency	Fixed	Male	0.16	1, 1086.9	0.36	0.55	
			Female	0.23	1, 1087.4	0.52	0.47	
			Male x Female	0.01	1, 1087.9	0.02	0.90	
		Random		Trial/Replicate	% var	Df	X ²	P
					2.11	2	8.37	0.01
					<i>MS</i>	<i>df</i>	<i>F</i>	<i>P</i>
Male Choice	Duration	Fixed	Male	79.74	1, 1084.2	3.12	0.08	
			Female	982.57	1, 1084.4	38.42	<0.0001	
			Male x Female	46.32	1, 1085.3	1.81	0.18	
		Random		Trial/Replicate	% var	Df	X ²	P
					3.86	2	21.25	<0.0001
					<i>MS</i>	<i>df</i>	<i>F</i>	<i>P</i>

Table C3. Full GLMM results for the hatchability and larval to adult viability of flies from parental and hybrid crosses.

<i>Response</i>	<i>Factor Type</i>		χ^2	<i>df</i>	<i>P</i>
Hatchability	Fixed	Cross Identity	19.1	5, 154	<0.01
	Random	Replicate Population	0.02	1	0.89
Larvae to Adult Viability	Fixed	Cross Identity	134.79	5, 154	<0.0001
	Random	Replicate Population	1.05	1	0.31

Table C4. Full LMM results for the development time and body size of flies from parental and hybrid crosses.

<i>Response</i>	<i>Factor Type</i>		<i>MS</i>	<i>df</i>	<i>F</i>	<i>P</i>
Development Time	Fixed	Cross Identity	4974.9	5, 154	987.18	<0.0001
	Random	Replicate Population		<i>Df</i>	<i>LRT</i>	<i>P</i>
				1	0.43	0.51
Body Size	Fixed	Cross Identity	0.01	5, 64	41.36	<0.0001
	Random	Replicate Population		<i>Df</i>	<i>LRT</i>	<i>P</i>
				1	1.41	0.23

Table C5. Full GLMM results for the fertility of female and male flies from parental and hybrid crosses.

<i>Sex</i>	<i>Response</i>	<i>Factor Type</i>		χ^2	<i>df</i>	<i>P</i>
Female	Proportion Red Eyed	Fixed	Cross Identity	67.49	5, 515	<0.0001
		Random	Replicate Population	0	1	1
Male	Proportion Red Eyed	Fixed	Cross Identity	19.47	5, 502	<0.01
		Random	Replicate Population	0	1	1