Research Article

Discoveries

Inversions Can Accumulate Balanced Sexual Antagonism: Evidence from Simulations and Drosophila *Experiments*

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Abstract

Chromosomal inversion polymorphisms can be common, but the causes of their persistence are often unclear. We propose a model for the maintenance of inversion polymorphism, which requires that some variants contribute antagonistically to two phenotypes, one of which has negative frequency-dependent fitness. These conditions yield a form of frequency-dependent disruptive selection, favoring two predominant haplotypes segregating alleles that favor opposing antagonistic phenotypes. An inversion associated with one haplotype can reduce the fitness load incurred by generating recombinant offspring, reinforcing its linkage to the haplotype and enabling both haplotypes to accumulate more antagonistic variants than expected otherwise. We apply a purpose-built forward simulator to examine these dynamics under a tradeoff between viability and male display. These simulations indeed generated long haplotypes where alleles of opposing effects occupied alternate chromosomal arrangements. Antagonism increases with time, and can ultimately yield karyotypes at predictable frequencies, and notable genotype frequency differences between sexes and between developmental stages. To test whether this model may contribute to abundant inversion polymorphism in *Drosophila melanogaster*, we tracked inversion frequencies in laboratory crosses to test whether they influence male reproductive success or survival. We find that two of the four tested inversions show significant evidence for the tradeoff examined, with *In(3R)K* favoring survival and *In(3L)Ok* favoring male reproduction. Additionally, all inversions show survival differences between sexes, and paternal success depends on maternal genotype. Based on this work, we expect that balancing selection on antagonistically pleiotropic traits may provide a significant and underappreciated contribution to the maintenance of natural inversion polymorphism.

1 Introduction

1.1 The Enigmatic Prevalence of Inversion Polymorphism

Inversions, DNA segments in reversed sequence order relative to the ancestor, have been a focus of population genetic study for their direct or indirect effects on recombination in natural populations for more than a century. In their most dramatic effect on meiosis, inversions generate aneuploid gametes when a crossover occurs between the interior of an inverted region and its non-inverted homolog (White 1973). As aneuploidy is often fatal in embryos or severely deleterious, this may translate to a fecundity cost for a parent heterozygous for an inversion; however, depending on a species' reproductive biology, such reproductive fitness effects may be mitigated if the zygotes terminate early or before significant parental resource investment. Alternatively, crossovers may be inhibited directly in regions heterozygous for an inversion, or avoided by achiasmy or segregation of the aneuploid gametes to polar bodies in female meiosis (Sturtevant 1921; Sturtevant and Beadle 1936; Krimbas and Powell 1992; Coyne et al. 1993; Gong et al. 2005). Regardless of the differences between these scenarios, effectively fewer recombinant offspring are produced by inversion heterozygotes.

Inversions may also have direct effects on fitness when disrupting a gene, a regulatory element, or other genomic structure like a topologically associated domain (Lupiáñez et al. 2015; McBroome et al. 2020), but many inversion arrangements are expected to be neutral in and of themselves (Said et al. 2018). So, in the absence of indirect selection on the recombination effects of inversions, inversion variants are expected to evolve similarly to single nucleotide mutations: to be selectively removed when the net direct effects are deleterious, to sweep when the effects are beneficial, to be fixed or lost in proportion to frequency when neutral, or to be selectively maintained when associated with a balanced trait. Given that a common effect of inversion heterozygotes is the deleterious generation of aneuploid gametes, and that studies of extant inversions and engineered inversions suggest that inversions may commonly have minimal effect on regulation or coding regions, many inversion rearrangements may confer either deleterious or neutral fitness consequences in the absence of association with other variants.

In contrast to the above predictions, several well-studied examples are known in which inversion variants are maintained at an intermediate frequency within a population, presumably due to the indirect effects of inversions on recombination. Simulation and analytic theory have demonstrated that inversions associated with a local population can reduce recombination between locally adapted variants and unfit migrants, and thereby increase in frequency by association with the fittest haplotype locally (Kirkpatrick and Barton 2006; Charlesworth and Barton 2018). Such locally adaptive inversions could potentially also link together favorable combinations of variants between epistatically interacting loci (Dobzhansky 1970; Schaeffer et al. 2003; Hoffmann and Rieseberg 2008). Unless migration rates are high, these models predict inversions to be at high frequency in their favored geographic range but at low frequency elsewhere. Alternately, chance associations of both an inversion and its standard arrangement counterpart with different recessive deleterious alleles may cause a form of overdominant selection, where heterozygotes carrying both main haplotypes experience none of the deleterious effects of a homozygous recessive. This associative overdominance can persist for extended periods, particularly when the rate of recombination between inversion arrangements is low (Zhao and Charlesworth 2016; Gilbert et al. 2020). This model predicts that such haplotypes will most readily accumulate in small populations, or when inversions that are initially rare accumulate recessive deleterious variants before the arrival of a linked positive variant or change in selection pressure. This may extend the polymorphic lifespan of an inversion, and remain stable in small populations, but is not expected to remain indefinitely stable as an intermediate frequency polymorphism in sufficiently large populations due to the greater population recombination rates allowing gene conversion and/or double-crossover events to generate haplotypes lacking deleterious variants. Inversions may also link drivers and enhancers in meiotic drive systems (Babcock and Anderson 1996; Mroczek et al. 2006; Courret et al. 2019), and these drive-associated inversions may persist at equilibrium frequencies in the presence of repressors (*e.g.* Bastide et al. 2022). In natural populations, it can be difficult to distinguish among these alternate hypotheses, and they are not always mutually exclusive.

1.2 Inversions in *Drosophila melanogaster*

Drosophila melanogaster is a widely studied organism in many fields of biology, including evolutionary and population genetics. Multiple polymorphic inversions segregate at meaningful frequencies in natural populations of *D. melanogaster* (Aulard et al. 2002; Kapun et al. 2016; Kapun and Flatt 2019), but even in this model system, it is not entirely clear which population genetic processes are most responsible for shaping their frequencies. In this species, achiasmy in males (Morgan 1910; John et al. 2016) and a lack of evidence for female fecundity costs for most inversions (Sturtevant 1921; Sturtevant and Beadle 1936; Coyne et al. 1993; Gong et al. 2005) could allow inversion rearrangements to have limited fitness consequences. Further, genome editing tools have allowed for the creation of synthetic inversions, and the finding that these structural rearrangements themselves are not responsible for the gene regulatory changes that are associated with natural inversions in *D. melanogaster* (Said et al. 2018). Instead, given that breakpoints of common *D. melanogaster* inversions occur disproportionately in regions that confer greater recombination suppression (Corbett-Detig 2016), the primary evolutionary relevance of these rearrangements appears to be the resulting reduction of recombinant offspring between otherwise highly recombining loci. Hence, *D. melanogaster* inversions would appear to be maintained for the sake of maintaining linkage associations between distant variants.

D. melanogaster originated in southern-central Africa, in seasonally dry Miombo and Mopane woodlands, and (potentially after adapting to a human commensal niche) expanded out across much of Africa and into the Middle East around 13,000 years ago, and into Europe around 1,800 years ago (Sprengelmeyer et al. 2020). With an ancestral N_e of around 2 million (Sprengelmeyer et al. 2020), *D. melanogaster* populations have high levels of standing diversity and high population recombination rates. Many *D. melanogaster* inversions have geographic clines repeated between several colonized regions (Mettler et al. 1977; Reinhardt et al. 2014; Kapun et al. 2016), and inversions often show geographic differences in frequency that exceed genome-wide differentiation between the same populations (*e.g.* Pool et al. 2017). It has therefore been suggested that local ecological adaptation may shape the distribution of *D. melanogaster* inversions (Kapun et al. 2016; Kapun and Flatt 2019). Indeed, it is difficult to explain their geographic differentiation without some type of spatially-varying selective pressures.

Yet, it is unclear whether ecological adaptation is a sufficient explanation for *D. melanogaster* inversion frequency patterns, for two reasons. First, most of the common inversions become less frequent as one moves away from the sub-Saharan ancestral range (or similar warm environments) into potentially more challenging, colder high latitude and altitude environments (Aulard et al. 2002; Kapun et al. 2016; Lack et al. 2016a; Pool et al. 2017; Kapun and Flatt 2019). As these inversions are all derived (being absent from closely related species), local adaptation theory would predict that they arose to protect local adaptation against ongoing migration, more likely in the novel environment if it is experiencing high levels of immigration (Kirkpatrick and Barton 2006). It is unclear why inversions would be needed to adapt to the species' ancestral environments, or why ancestral "standard" arrangements would have an ecological advantage in derived environments that are very different from the ancestral range.

Second, even in the locations where inversions would hypothetically be ecologically advantageous, they rarely surpass intermediate frequencies (Pool et al. 2017; Sprengelmeyer et al. 2020). Under a local adaptation model, that observation would require a secondary explanation, such as very high levels of long distance gene flow or the fixation of recessive deleterious variants on inverted haplotypes, generating an associative overdominance at linked neutral loci (Frydenberg 1963; Sved 1968; Ohta and Kimura 1970; Zhao and Charlesworth 2016; Gilbert et al. 2020) that resists fixation. While migration may well limit inversion frequencies in some populations, we note that some putatively adaptive Single Nucleotide Polymorphism (SNP) variants do achieve much greater frequency differentials between populations (da Silva Ribeiro et al. 2022), and thus we would have to suppose that inversions are under relatively weaker selection to explain their lesser differentiation. Regarding the hypothesis of associative overdominance, we note that *D. melanogaster* populations do contain substantial recessive genetic load (Greenberg and Crow 1960), and it has been speculated that associative overdominance might boost inversion frequencies in *D. melanogaster* populations during founder events (Pool et al. 2012) or in isolated wilderness environments (Sprengelmeyer et al. 2020). However, it seems likely that in large human-commensal populations, *D. melanogaster* inversions would escape from linked deleterious variants through gene conversion or double crossover events with standard chromosomes, in light of both the antiquity of most of these inversions (Corbett-Detig and Hartl 2012) and the very high population recombination rates of *D. melanogaster*. Homozygous lines

have also been generated for each common inversion, demonstrating a lack of perfect linkage of the inversions with recessive lethal or infertile variants (*e.g.* Lack et al. 2016a).

While local adaptation, gene flow, and associative overdominance may all contribute to geographic variation in inversion frequency among *D. melanogaster* populations, it is not clear that these processes are collectively sufficient to explain observed patterns. Alternatively, the allele frequencies and diversity patterns of at least some of these inversions might be primarily shaped by some form of balancing selection acting on inversion-linked variation, in which the balanced frequency is dependent on the environment (Kapun et al. 2023). Balancing selection on inversions has received increasing attention in recent literature (Wellenreuther and Bernatchez 2018; Faria et al. 2019). However, there has not been a proposed mechanism whereby alleles at multiple linked loci undergoing balancing selection would directly benefit from linkage and thereby maintain an associated inversion polymorphism under indirect selection. Here we propose one such mechanism, in which alleles that contribute to naturally frequency-dependent traits like competitive mating display simultaneously contribute antagonistically to another trait, such as the survival to reproductive maturity.

1.3 Sexually Antagonistic Polymorphism in *D. melanogaster*

Among mechanisms of selection, sexual selection is both prevalent, and provides a simple mechanism for frequency dependence (O' Donald et al. 1997). Alleles that contribute to mate choice systems may often follow a negative frequency dependence within the population, as display success depends on the relative quality of a male among local competitors, and the more common a display-enhancing allele is, the fewer competitors it succeeds over. Combinations of display-enhancing alleles may similarly gain a disproportionate benefit together, as male mating success in competitive display systems can be skewed towards a few successful individuals (Bateman 1948; Jones et al. 2002; Tatarenkov et al. 2008, though see Gowaty et al. 2012; Hoquet et al. 2020) such that the last few alleles to push display into the top quantile contribute a significantly greater fitness increase to the genotype than the first few display alleles. This interaction of alleles is a form of epistasis - a non-independent fitness interaction between loci - that is emergent and non-specific to the genetic identity of the variants. In other words, the alleles

150 contribute independently to the generation of a trait, and the fitness of the individual is a nonlinear function of
151 that trait (Blows and Brooks 2003; Rest et al. 2013; Otwinowski et al. 2018). This epistatic interaction suggests a
152 potential for indirect selection on the recombination-suppressing effects of inversions, in order to maintain
153 combinations of sexually antagonistic variants that have the greatest fitness when carried together. The tendency
154 of selection to favor the reduction of recombination when the haplotypes present are at a fitness optimum is well
155 studied, and has been termed the 'reduction principle' (Altenberg et al. 2017).

156 While the emergent epistasis generated by a mate choice system may favor linkage of variants, alleles that
157 contribute only to a sexually selected display are not expected to remain as balanced polymorphisms, rather they
158 are expected to fix, albeit perhaps slowly as their fitness benefits diminish at high frequencies. However, if these
159 alleles simultaneously contribute deleteriously to other components of fitness, they are pleiotropic (Stearns 2010),
160 and given the sex-limited benefits of the display they would likely also be sexually antagonistic (Rowe et al. 2018),
161 whereby traits increase the fitness of one sex but decrease fitness if found in the other. Pleiotropy can result from
162 traits that are inherently physiologically coupled, from resource allocation restraints, or from the specific role or
163 behavior of genes. Pleiotropy can be sexually antagonistic when the traits in the tradeoff are differentially
164 important to different sexes, which may be common given the differing fitness requirements of the sexes in a
165 population.

166 *D. melanogaster* mating involves a male display performance and female acceptance or rejection. A number of
167 experimental evolution studies (Rice 1992; Rice 1996; Audet et al. 2023) and genetic studies (Innocenti and
168 Morrow 2010; Cheng and Kirkpatrick 2016; Glaser-Schmitt et al. 2023) have confirmed the prevalence of sexually
169 antagonistic loci in *D. melanogaster*. Pleiotropic alleles that trade off between survival and male display quality
170 may be the simplest case of antagonism to consider for a model of balancing selection, and one highly relevant to
171 the biology of *D. melanogaster*.

172 Given the relevance of sexual selection and sexual antagonism to *Drosophila* evolution, we propose that
173 polymorphic *D. melanogaster* inversion and standard arrangements may help maintain haplotypes of alleles that
174 specify opposite ends of a sexually antagonistic tradeoff. We first explore this model through forward simulation,

to establish that it may in theory arise in a population and maintain inversion polymorphism over a significant number of generations. Then we examine the results of an experiment with an outbred laboratory population of *D. melanogaster*, to test whether common inversions show changes in frequency over the course of a generation in a manner consistent with a trade-off between survival and male reproductive success. Many such antagonistically pleiotropic scenarios are possible under different selective tradeoffs, but we focus on a specific scenario of sexual antagonism here.

2 Results

2.1 A Model of Sexually Antagonistic Inversions

Here, we consider a population featuring a competitive mate choice system that results in a skewed distribution of reproductive success among males. Within this population, we model a genomic region containing a number of linked loci, each with alternate antagonistic, pleiotropic alleles that trade off between male display success and survival likelihood (of females or both sexes). If mate choice is highly competitive, then superior display quality can enable males to mate successfully in excess of other display phenotypes they compete against. This gives a disproportionate benefit to additional mate competition quality if it allows a male to out-compete others, while additional survival costs stay proportional. Yet, fitness conveyed by display quality is also frequency-dependent; since mating success depends on the display qualities of other males, the relative advantage of a display trait will be diminished as more males carry it. And so, for an antagonistic variant that benefits display and begins under net positive selection, the benefit will decrease as the variant rises in frequency, until eventually it is equal in magnitude (across both sexes) to its pleiotropic survival cost – at which point an equilibrium frequency is reached. At this stage, the highest fitness among haplotypes may favor either of two extreme strategies - either producing among the most reproductively competitive males (by carrying a maximal number of antagonistic variants and paying the necessary cost of survival likelihood) or else paying none of the survival cost (at the expense of male reproductive competitiveness, carrying none of the antagonistic variants). In these conditions, we propose that

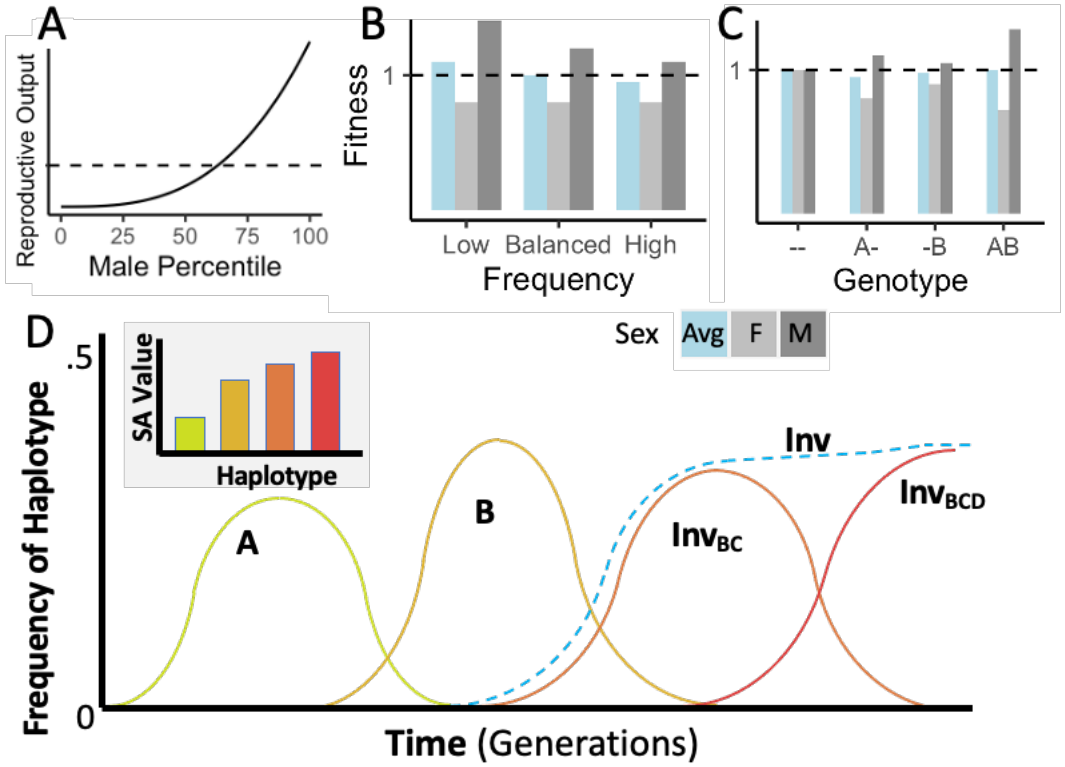
198 selection will generate linkage disequilibrium between sets of associated alleles that together strongly favor either
199 display or survival, and selection will favor the association of these haplotypes with alternate inversion karyotypes
200 to reduce the recombination load experienced in heterozygous parents carrying one haplotype each from the
201 extreme survival- and display-focused classes. We further predict that in a population that is close to the balanced
202 equilibrium state, the population of adults will contain a higher frequency of survival-focused haplotypes than the
203 population of zygotes, but the display-focused haplotypes will increase in frequency in zygotes relative to adults.

204 To build an intuition for the model, let us consider a simplistic model population with mate choice and phenotypic
205 variation in male display. Particularly if each female can choose amongst several or many males, the variance of
206 offspring number among males (Fig 1A) may be much higher than for females - yielding a relatively convex
207 distribution in which the least and most successful males produce no or many offspring respectively. A genetic
208 change that increases reproductive quality in a male can increase competitiveness and offspring count, but an
209 increase in the display of a male with low quality (change i in Figure 1A) results in a smaller change in number of
210 offspring than an increase in display of a male with high quality (change ii in Figure 1A), due to the reproductive
211 skew towards highly competitive individuals. Therefore, multiple variants increasing the reproductive rank of an
212 individual together have a larger fitness benefit than the combination of each substitution separately - a positive,
213 synergistic epistatic effect.

214 And yet, as pointed out above, this positive epistasis is accompanied by a negative frequency dependence due to
215 an antagonistic variant's decreasing advantage over other males as its frequency rises and more competing males
216 carry it. Display-enhancing variants with little or no pleiotropic cost may ultimately fix in spite of their diminishing
217 advantage as a sweep progresses, whereas those with relatively high pleiotropic costs (modeled here as reduced
218 survival probability) may be unconditionally deleterious and lost quickly. However, pleiotropic variants with more
219 comparable fitness effects on display enhancement and survival reduction may end up at a balanced equilibrium
220 frequency: when rare the allele experiences a large benefit from its display effects and is expected to increase in
221 frequency, but above a certain frequency the benefit becomes less than the antagonistic cost (Figure 1B) and the

allele is expected to decrease in frequency. In the absence of other forces, this dynamic is expected to maintain the allele under balancing selection indefinitely.

Consider a haploid population with two linked loci that carry such antagonistically pleiotropic alleles. Further consider the unrealistic circumstance that all alleles are at a frequency of 0.5 with perfect linkage equilibrium, and so all possible haplotypes are present at a frequency of 0.25. The haplotypes with all display-favoring alleles will then tend to be disproportionately chosen over other haplotype classes during male reproductive competition, yielding a positive epistatic interaction as seen in Figure 1A. If selection proceeds without recombination, the most display-heavy haplotype increases in frequency until reaching an equilibrium at which its relative advantage in mate choice is reduced enough to be balanced by the pleiotropic survival cost, which is assumed to be frequency-independent and non-epistatic among loci (Figure 1B). In this new state, the fitness of the most display-heavy haplotype and its opposite survival-focused haplotype are equally fit. However, haplotypes of intermediate composition all have lower fitness: they experience some pleiotropic cost, but due to the reproductive skew of mate choice they are selected disproportionately less often than the display-heavy haplotypes, so the net fitness contribution is negative (Figure 1C). Therefore, recombination between the two extreme haplotypes will generate less fit haplotypes in the gametes, and a local modifier of recombination that reduces this cost of recombination, like an inversion, will be favored by indirect selection (Feldman et al. 1980; Otto and Lenormand 2002). As inversions reduce recombinant gametes between haplotypes heterozygous for the inversion, an inversion introduced to this population should benefit from associating with one or the other extreme haplotype and should then increase to the balanced frequency of that haplotype.



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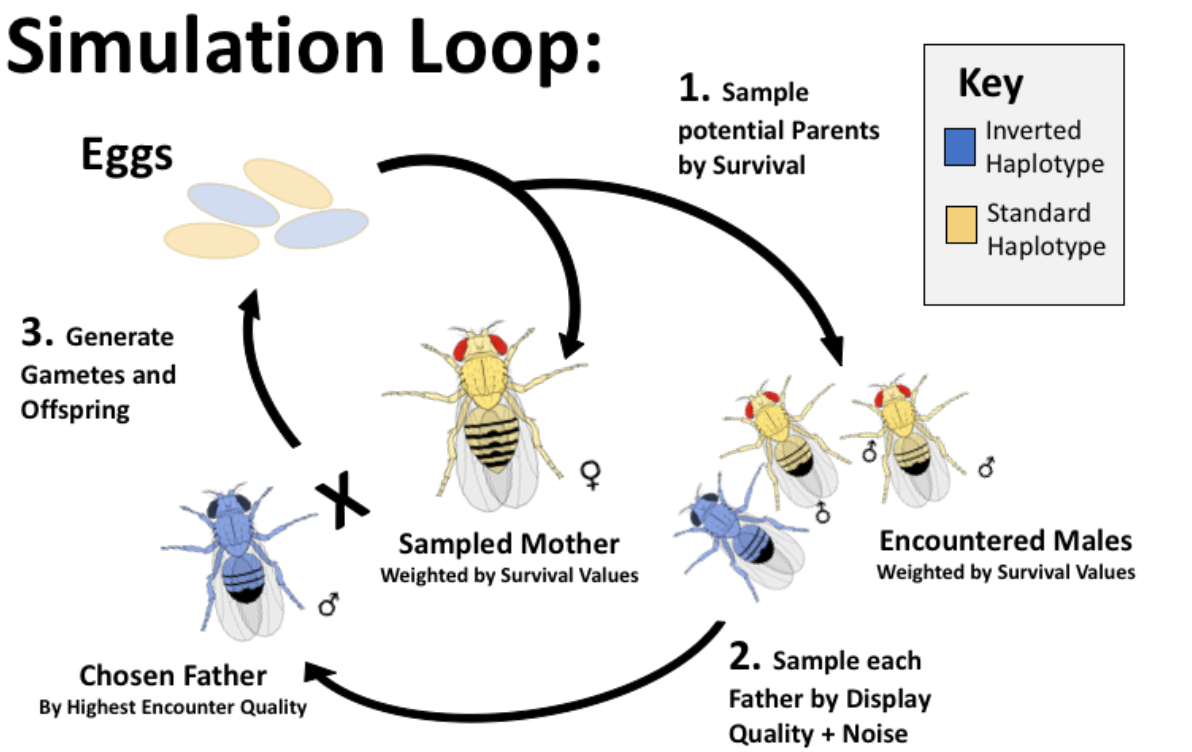
242 [Figure 1: Conceptual representations of the proposed model of inversion-associated balanced sexual antagonism.
243 (A) Hypothetical skewed distribution of male mating success, yielding a greater variance in reproductive success for
244 males than for females in our model. (B) Fitnesses of display-favoring alleles under sexually antagonistic balancing
245 selection, illustrating a pleiotropic variant that should rise in frequency when rare, but decline if frequency exceeds
246 the balanced equilibrium value. (C) Fitnesses of display-favoring alleles at two haploid loci under the same model,
247 illustrating synergistic epistasis between displaying-favoring alleles. (D) A hypothetical trajectory of four such
248 mutations, in which B outcompetes A, an inversion links B and C to create a more strongly display-favoring
249 haplotype, and then the addition of D to that haplotype furthers the accumulation of antagonistic variants,
250 reaching an equilibrium frequency while displacing less extreme display-favoring haplotypes.]

2.2 Simulation Results

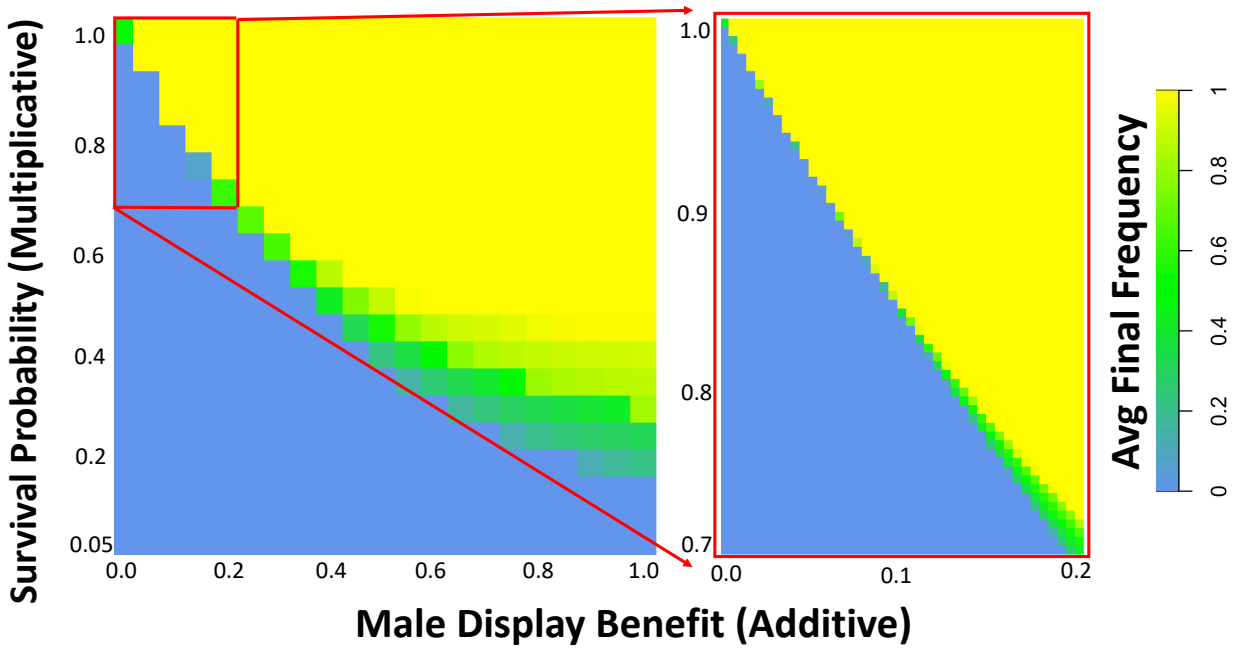


[**Figure 2:** The layout of a single simulated generation. Simulated individuals each reach the reproductive stage with probability in proportion to the product of the survival effects of their alleles. To generate each offspring in the next generation, a surviving female is first randomly sampled (with replacement) to be the mother. Then a specified number of surviving males are randomly sampled to generate the pool of males encountered by the sampled female - without replacement for each pool and with replacement between pools. For each encounter pool, each male is assigned an observed display quality as the sum of the display effects of its alleles, plus a normally distributed noise effect for each encounter. The highest quality male in the encounter pool is selected as the father, and the offspring genome is generated with crossover and gene conversion events, where odd numbers of crossovers in the interior of an inversion are thrown out and resampled from the same parent. In the

262 simulations presented, males do not recombine when generating gametes, in light of the biology of *D.*

263 *melanogaster*.]

264



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266 [**Figure 3**: Simulations show that a balanced equilibrium frequency exists for certain sexually antagonistic variants.

267 The average final frequency of a single mutation with given effects on survival proportion (of both sexes) and male

268 reproductive quality score is shown, with green indicating intermediate balanced frequencies. These simulations

269 involve a single antagonistic variant with initial frequency 0.5, in a population of 1,000 individuals simulated for

270 20,000 generations, with further details as indicated in the Materials and Methods. The left panel depicts trait

271 values at 0.05 increments, whereas the insert presents a finer-scale set of simulations, every 0.005 trait units.]

272

273 We investigated the predictions of the above model of inversion-associated balanced sexual antagonism using a

274 purpose-built forward "Sexually Antagonistic Inversion simulator" (SAIsim; see Materials and Methods). This

275 simulation program incorporates mutation of new nucleotide and inversion polymorphisms, crossing over and
276 gene conversion (including between chromosomes heterozygous for inversions), drift, and sexually antagonistic
277 selection (Materials and Methods). SAIsim allowed us to model the influence of polymorphic inversions on
278 recombination, as well as the changes in linkage between sites entailed by the mutation and fixation of an
279 inversion. It explicitly models mate selection, in that each female randomly selected to reproduce is assumed to
280 encounter a number of randomly selected males, and the display values of each male are assigned based on the
281 sum of genetic and environmental effects (with genetic effects based on genotypes at antagonistic loci), and the
282 male with the highest display value then succeeds in this mating (Figure 2). We found it easiest to write a new
283 simulator as no extant simulator accurately managed mutation and recombination of multiple inversion
284 polymorphisms, as well as an explicit mate competition in generating offspring.

285 With this simulator, we first confirmed that individual variants with an antagonistic pleiotropy between survival
286 (initially of both sexes) and male reproduction can generate stable selectively balanced polymorphisms *in silico*.
287 We simulated populations of N = 1000 carrying a single variant of a specified survival and display quality at an
288 initial frequency of 0.5. After 20N generations, we calculated the average final frequency of the variant. From this
289 analysis we can see that there is a range of paired trait effects for which the mutation remained at intermediate
290 frequency after 20N generations (Figure 3). In a scenario where only females pay the survival cost, the balanced
291 parameter space is shifted somewhat, but qualitatively the results are similar (Figure S1). Alleles with small effect
292 sizes had only a narrow band of balanced selection, outside of which they were either fixed or lost, while large-
293 effect alleles remained balanced across a larger area of simulated parameter space. While this balanced range is
294 narrow, such variants may persist under balancing selection indefinitely in the absence of other forces.

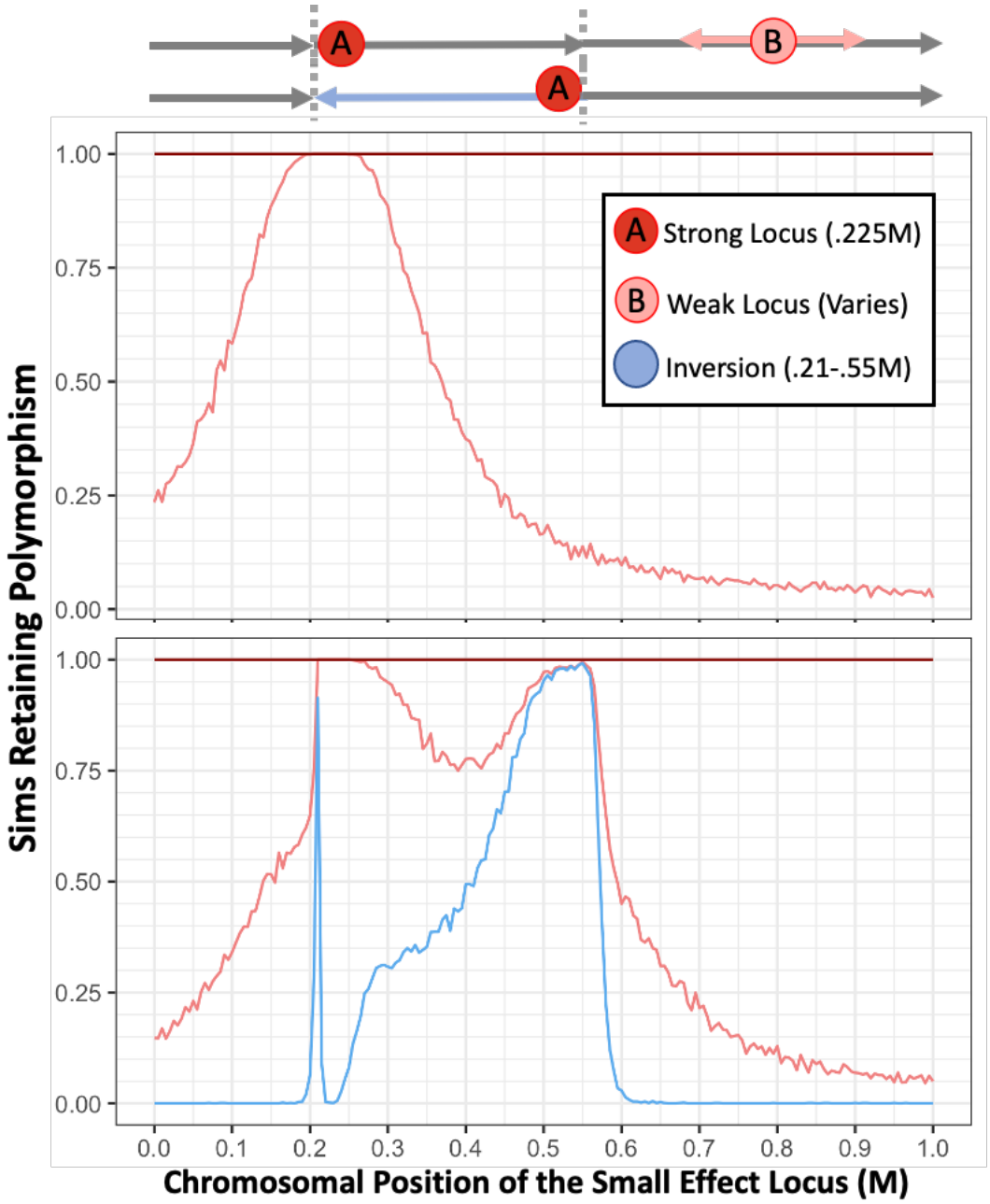
295 To test the effects of association with an inversion, we initially ran two sets of simulations, considering two
296 antagonistic variants found to undergo balancing selection separately. First, we spaced the two antagonistic loci at
297 a varying genomic distances, in the absence of an inversion (Figure 4). In each case, the stronger antagonistic
298 variant (modelled at fixed position 0.225M) persists, and so we focus on whether the weaker variant (at varying
299 positions) also persists with it, or else is outcompeted by the more antagonistic variant. As seen in the upper panel

of Figure 4, the weaker variant (pale red line) is retained at close linkage, as the haplotype containing both antagonistic variants acts as a single larger effect supergene. However, as the weaker variant moves farther from the stronger variant, recombination increasingly generates unfit intermediate genotypes compared to the extremes of the reproduction-survival tradeoff, and the stronger variant outcompetes the weaker variant among these haplotypes, leading to the loss of the weaker variant.

We then examined the same spacings between antagonistic variants across a region containing a polymorphic inversion, with its left breakpoint close to the stronger variant, and the right breakpoint within the range of the positions in which the weaker variant was simulated (Figure 4, lower panel). When the inversion is added, the weaker variant is now retained when the two antagonistic variants are relatively more distant, due to association of the weaker variant with the right-hand inversion breakpoint (pale red line), at which point this association also preserves the inversion itself in a polymorphic state (light blue line). While more stochastic, a weaker antagonistic variant near the middle of the inversion also appears to maintain polymorphism more frequently in systems starting with an inversion than without, i.e. across approximate positions 0.30-0.45M in Figure 4, in spite of its weaker linkage with the inversion itself due to double cross-over events. The inversion is not maintained when the weaker antagonistic variant is within the inversion but tightly linked to the stronger variant (see positions 0.22-0.25M); here there is little crossing-over for an inversion to suppress, and so the effect of the inversion approaches neutrality. Interestingly, there is a small window of weaker variant position in which the inversion maintains polymorphism even in the face of relatively tight linkage to the stronger variant (see positions 0.2-0.22M). Here, the weaker antagonistic variant is more tightly linked to the breakpoint than to the stronger variant in this range, and presumably benefits more consistently from the crossover reduction. The maintenance of the weaker variant drops more rapidly outside of the inversion than over similar distances in the simulation without inversions (positions 0-0.2M, bottom versus top panel). This seemingly counter-intuitive result may stem from crossover events that result in aneuploid gametes being resampled in our simulations, which effectively increases the crossover rate outside the inversion breakpoints, a behavior which should be biologically reasonable in many clades. Hence, for a weaker variant outside the left inversion breakpoint, the presence of the inversion can

325 increase the crossover rate between the two antagonistic variants, and thus reduce the probability of of the

326 weaker variant surviving by retaining linkage to the stronger one.



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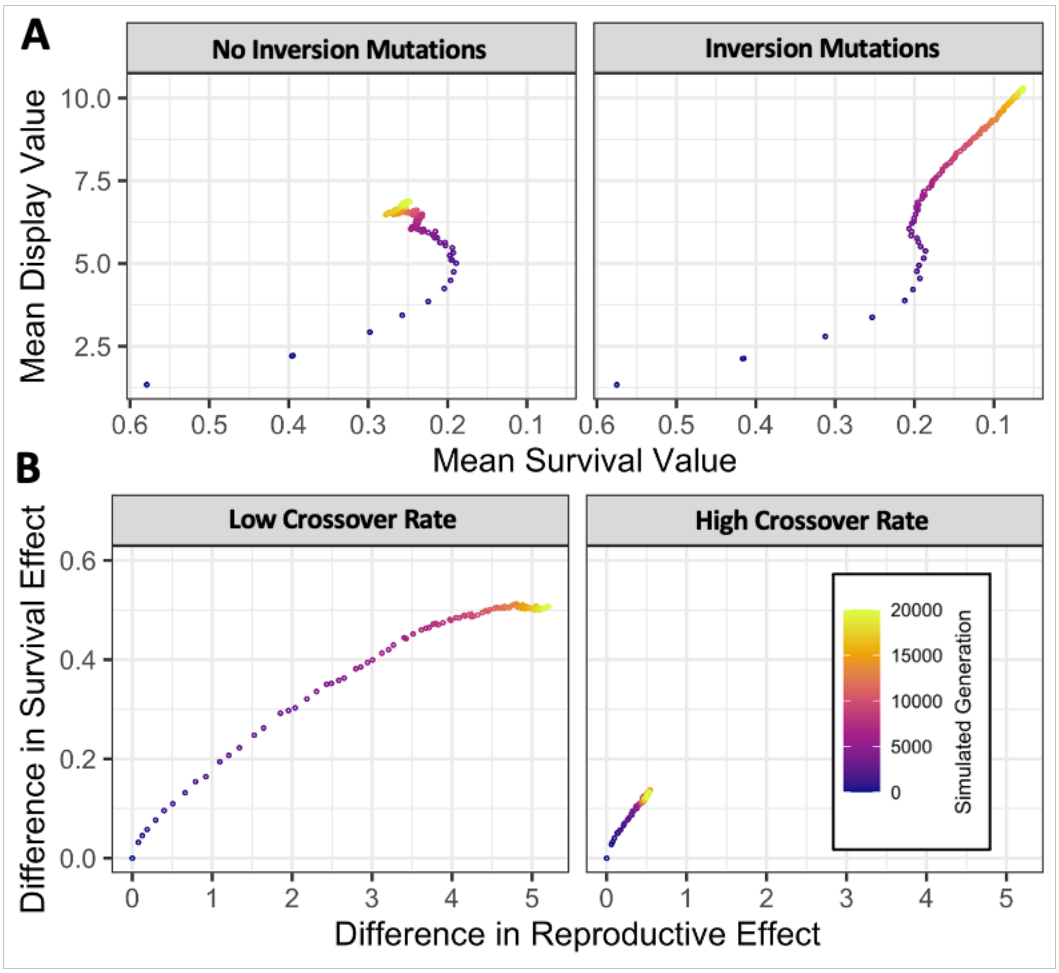
328 [Figure 4: Simulated inversions persist as polymorphisms when linked to sexually antagonistic, pleiotropic variants.

329 In turn, the presence of inversions facilitates the accumulation of antagonistic variation. Results of simulations

with two defined antagonistic variants alone (upper panel) and in the presence of a defined inversion (lower panel). The 1 Morgan chromosomal segment is diagrammed above the plots, with the inversion breakpoints marked. The proportion of simulations in which the second, variably-positioned weaker antagonistic variant (survival and reproductive values 0.86, 0.12) maintains polymorphism is shown (light red line), dependent on the recombination distance from a stronger allele (0.75,0.3) at fixed position 0.225M, which always persists (dark red line). In the lower panel, the proportion of replicates in which the inversion is retained is also plotted (blue line). Populations start with an equal frequency of all haplotypes in a population of 1000 individuals. 1000 replicate simulations were run for 20*N* generations in each parameter combination.]



340 [**Figure 5**: Simulated randomly mutating inversions persist as polymorphisms and accumulate sexually antagonistic
341 variation when linked to sexually antagonistic, pleiotropic variants. (A) In simulated populations with sexually
342 antagonistic mutations, simulations which also allow inversion mutations accumulated more average display
343 benefit and survival cost across generations (right panel) than those without inversions (left panel). (B) When
344 inversion mutation is enabled, a low crossover rate (left panel) allows populations to accumulate larger differences
345 between the most common karyotype and all others than when the rate of crossover is particularly high (right
346 panel). 1000 replicate simulations were run for 20*N* generations in each parameter combination.]

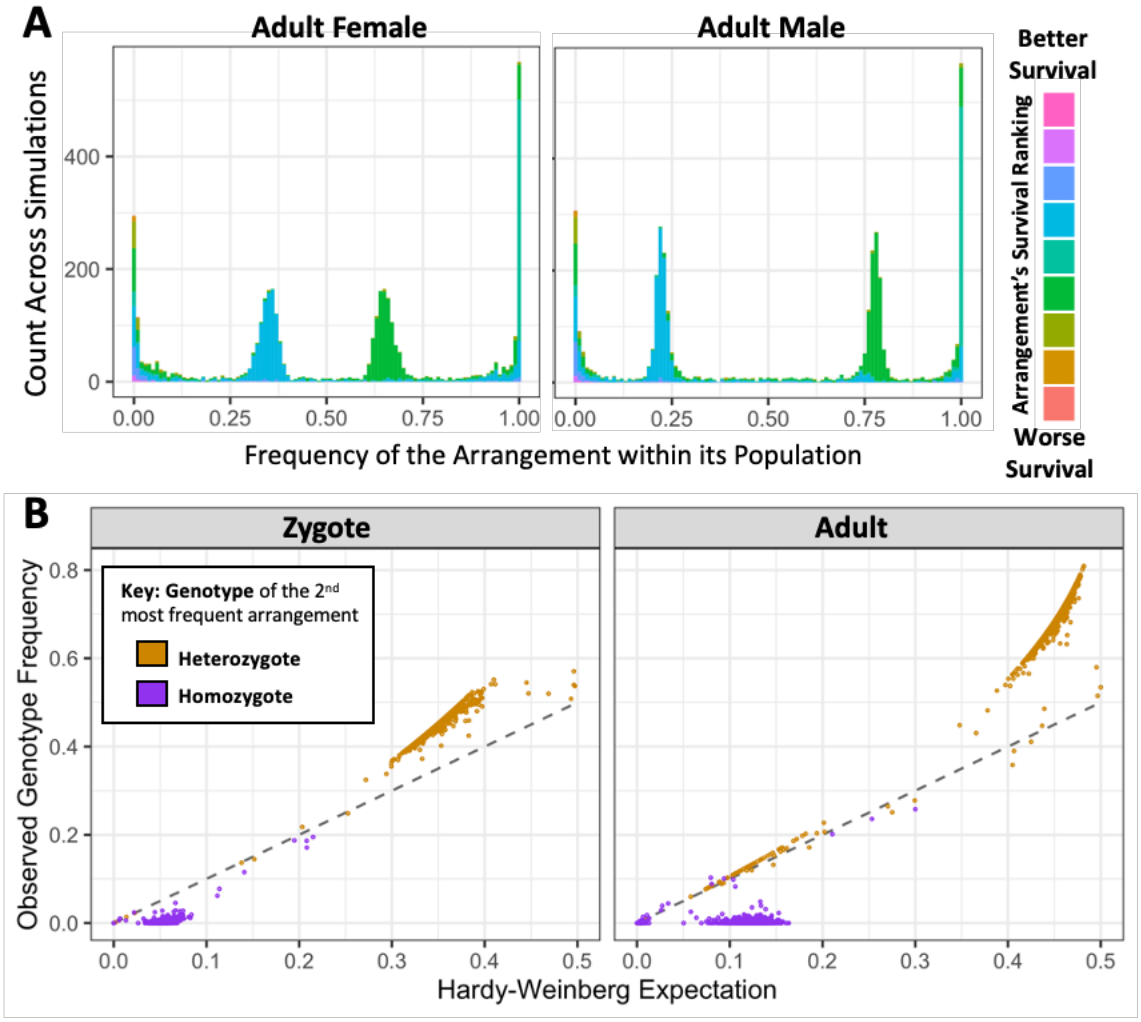
347

348 In further simulations, we began with a population initially free of variation, in which sexually antagonistic
349 mutations arise at a specified rate, randomly located across a 1M simulated region. These mutations had
350 pleiotropic effects increasing reproductive quality score and decreasing survival proportion, each drawn separately
351 from distributions (Materials and Methods). Depending on the effect scores drawn, a mutation could be broadly
352 advantageous (potentially leading to a selective sweep), broadly deleterious, or potentially subject to balancing
353 selection (Figure 3). Inversions (of random lengths and positions) also arose at a specified rate. The frequency
354 trajectories of antagonistic variants and inversions are tracked, and the simulation was run for 20*N* generations to
355 allow near-equilibrium patterns to be established. These simulations established the potential of a population to
356 mutate and evolve an inversion that links sexually antagonistic, pleiotropic alleles to maintain polymorphism in a
357 balanced manner (Figure 5B), although the degree of antagonism accumulated depends strongly on the rate of
358 crossing over (relative to the occurrence of antagonistic mutations; Figure 5C).

359 The simulations that successfully maintained balanced antagonistic chromosomes also had an interesting
360 distribution of allele and genotype frequencies of the balanced haplotypes at equilibrium. Without any selection,
361 the inversion frequency follows a pattern characteristic of the expected neutral site frequency spectrum (Figure
362 S2). However, in parameter spaces where simulations with shared survival costs between sexes maintained
363 polymorphism under balancing selection, inversion frequency distributions were tightly clustered around an
364 intermediate frequency (Figure S2, 6A). Strikingly, the frequencies of alternate haplotypes consistently clustered

365 around 0.25 and 0.75 among the zygote population. We found that this outcome was related to the genotype
366 frequencies of reproducing adults. These simulations evolve toward strong antagonism, and it is predominantly
367 males homozygous for the display-favoring haplotype (whether that is inverted or standard) who succeed in
368 reproducing, and these males therefore contribute one copy of the display-favoring haplotype to each of their
369 male and female offspring. However, this haplotype's significant cost to viability and longevity would select for the
370 heterozygous females over the display-favoring homozygotes. Given that successful parents would predominantly
371 be heterozygous females and display-homozygous males, three quarters of transmitted chromosomes have the
372 display-favoring haplotype and one quarter does not, yielding the 0.25 and 0.75 inversion frequencies seen in
373 simulations.

374 The sex-specific fitness dynamics present at equilibrium under this model also result in population-wide
375 genotype frequencies that depart from standard Hardy-Weinberg expectations. If the preponderance of matings
376 are between heterozygous females and display-favoring males, then homozygotes for the survival-focused
377 haplotype will rarely be produced (as confirmed in Figure 6B). We note that in cases where the standard
378 arrangement favors male display, then especially if antagonism is strong, this model could explain an observed
379 deficiency or absence of inversion homozygotes, even if the inversion was not associated with any recessive
380 deleterious variation. In contrast, heterozygotes may become disproportionately frequent after viability selection
381 (Figure 6B). And yet, this heterozygote abundance reflects neither the existence of recessive deleterious variants
382 on each haplotype, nor overdominance with respect to viability. Hence, our model may offer an alternative
383 explanation for deviations from Hardy-Weinberg genotype frequencies observed for polymorphic inversions in
384 natural populations.



[**Figure 6:** Antagonism-associated karyotypes reach predictable equilibrium frequencies in simulations with antagonistic and inversion mutations, when survival costs are shared between sexes. (A) Histograms of the number of arrangements at a specified frequency across all simulation replicates in females (left) and males (right), colored by how the average survival effect of that arrangement ranks compared to the others in its population. Ranks are normalized to be relative to the median arrangement value, to better account for rare arrangements and ties. These simulations reach an equilibrium with two predominant arrangements, with the more survival-focused arrangement around an overall frequency of 0.25 and more common in females, and the more male-competition-focused arrangement around a frequency of 0.75 and more common in males. (B) Because successful males tend to be homozygotes and successful females heterozygotes, the less frequent of the two major arrangements (which

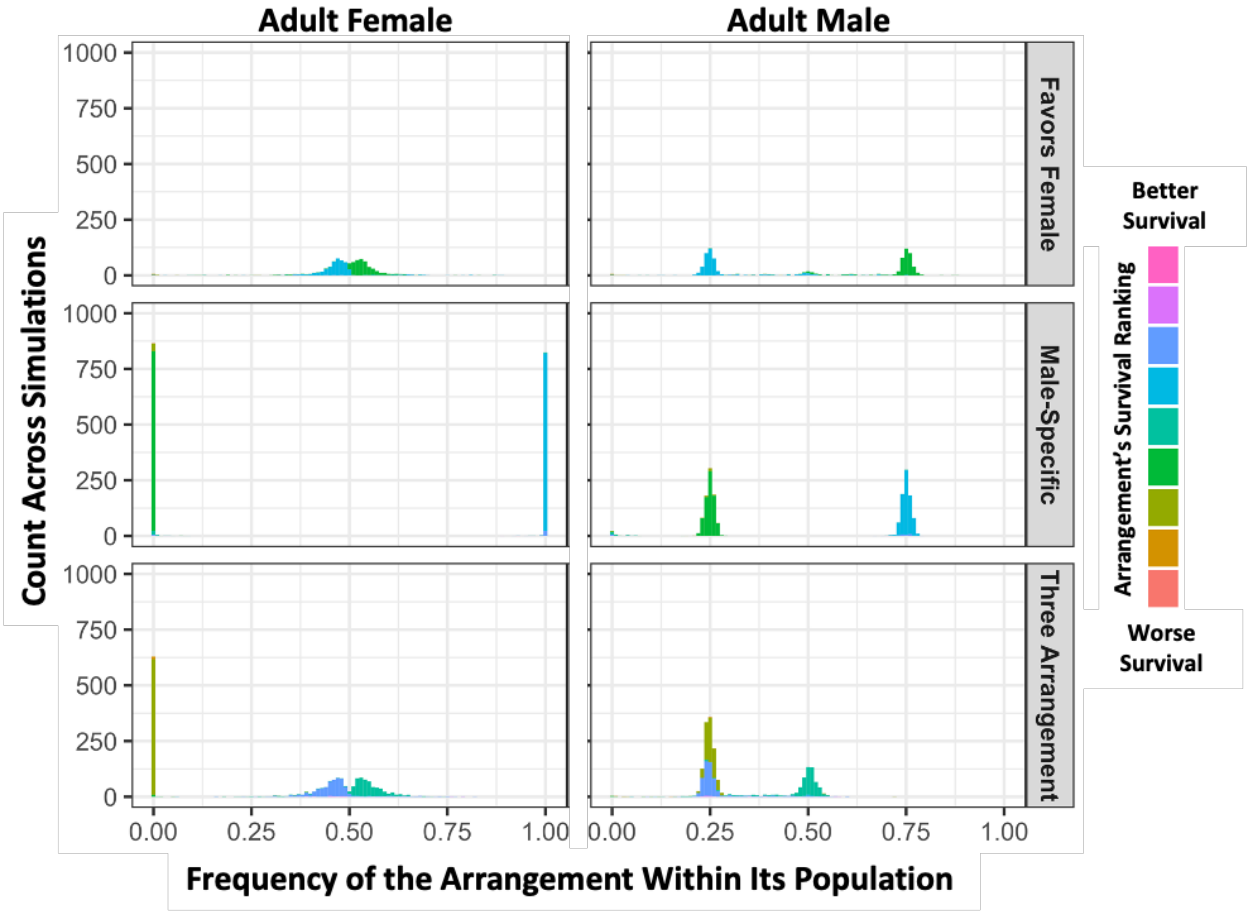
395 tends to favor survival) has homozygote frequencies far below the Hardy-Weinberg expectation, and there is an
396 excess of heterozygotes. These genotype frequencies shift somewhat between zygote (left panel) and adult (right
397 panel) populations as the genotype affects survival.]

398 We further ran simulations in which the survival costs were not shared, but instead specific to females,
399 representing a distinct, stronger form of sexual antagonism. In these simulations, we surprisingly recovered three
400 different equilibrium arrangement states (Figure 7). 20.0% of simulations exhibited a state similar to the shared
401 costs simulations presented in Figure 6, where we infer that fathers were homozygous for a Z-like display-favoring
402 arrangement, and mothers were heterozygous and carried a W-like survival-focused arrangement that is observed
403 in surviving but not reproducing males. 37.0% of simulations also developed two predominant arrangements, but
404 with Y-like instead of W-like dynamics. In these cases, we infer that the mothers were homozygous for an X-like
405 survival-focused arrangement, but the fathers were heterozygous and carried a Y-like display-focused arrangement
406 that was apparently lethal in females. 40.6% of simulations instead featured three intermediate frequency
407 haplotypes: with both Y-like and W-like arrangements restricted to the fathers and mothers respectively, and a
408 third shared arrangement with intermediate phenotypic effects.

409 Specific properties of our simulations may have influenced the occurrence of these three stable states under the
410 female-limited cost model. To reduce the computational complexity of both crossovers and arrangement-specific
411 trait values, the simulations do not allow two inversions on the same individual chromosome, whether overlapping
412 or not, though two chromosomes sampled from the population may have distinct inversions with overlapping
413 breakpoints. This means that if two arrangements, each with an inversion, become the prevalent haplotypes and
414 thereby remove all standard arrangement chromosomes from the population, there is no mutational path to
415 generate a third arrangement. However, a portion of the two-state simulations still had the ancestral, non-inverted
416 arrangement as one of the two haplotypes present, which could have been mutated to a third arrangement to
417 establish the three-arrangement pattern. We are not sure what selective or mutational barriers may prevent
418 transitioning between each state once established. One clear barrier is that it is difficult to generate a less extreme

419 arrangement from a Y-like arrangement, as these can accumulate an indefinite number of mutations since males
420 experience no costs.

421



422

423 [**Figure 7:** In simulations with female-limited survival costs, three distinct outcomes are observed involving either
424 two or three balanced haplotypes. From simulations with randomly occurring antagonistic and inversion
425 mutations, histograms of inversion frequencies across simulations for surviving females (left) and males (right) are
426 partitioned into three categories based on sex-specific haplotype frequency outcomes. The "Favors Female"
427 category (top) includes simulations in which there are exactly two arrangements with frequencies between 0.1 and
428 0.9 in both sexes (20.0% of replicates). The "Male-Specific" category (middle) includes simulations in which there
429 are two arrangements with whole-population frequencies between 0.1 and 0.9, but one arrangement is absent in

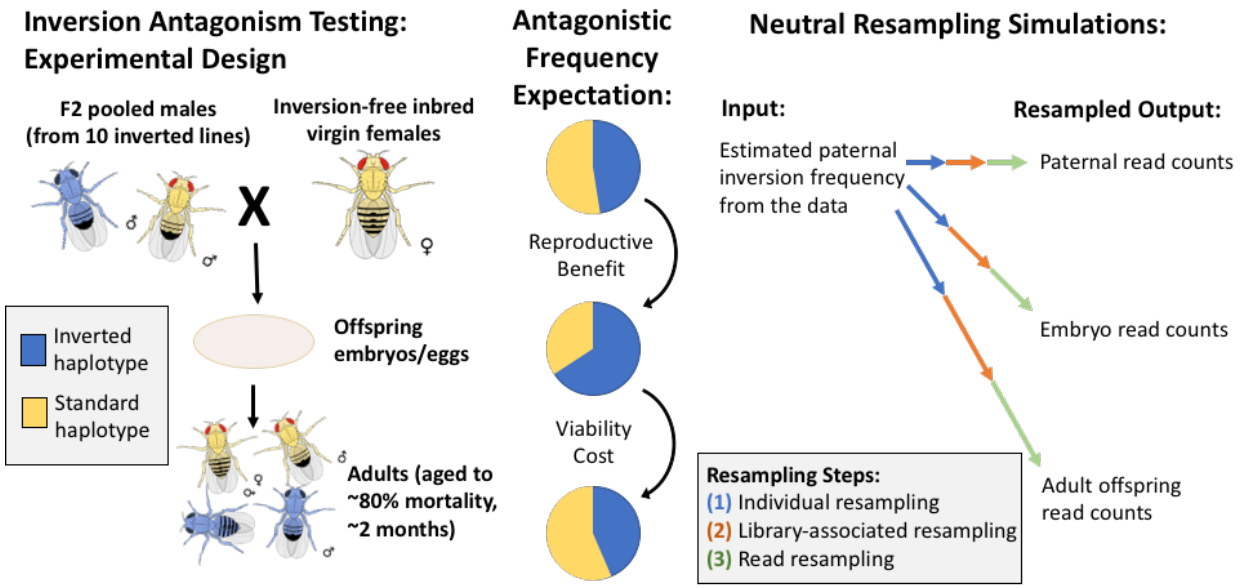
adult females (37.0% of replicates). The “Three Arrangement” category (bottom) includes simulations in which there are exactly three arrangements with whole-population frequencies between 0.1 and 0.9 frequency (40.6% of replicates). 2.3% of simulations did not fit in these categories and had only one predominant arrangement. As in Figure 6, inversions are colored by how the average survival effect of that arrangement ranks compared to the others in its population, and ranks are normalized to be relative to the median arrangement value, to better account for rare arrangements and ties.]

2.3 Empirical tests for inversion-associated tradeoffs

A specific motivation for developing and evaluating the model of inversion-associated, balanced sexual antagonism was to potentially better explain the pattern of inversion frequencies observed in *D. melanogaster*, in which multiple inversions reach some of their highest frequencies in populations from the ancestral range or with similarly tropical/subtropical climates. We therefore set out to test experimentally whether any of four inversions common in a Zambia population of this species show evidence for tradeoffs between male reproductive success and survival. We assembled an outbred population with moderate frequencies of each inversion to obtain karyotypically diverse males. We set up four crosses using these males along with females from one of four different inversion-free inbred Zambia lines. This crossing scheme is represented in Figure 8. These parental crosses occurred in a single large population cage, with populations of at least 600 of each sex. We developed a PCR and next-generation sequencing-based assay to estimate inversion-associated SNP frequencies in each pool of males and in each corresponding pool of embryos. These SNPs (given in Table S1) were based on our own analysis of variants that showed perfect association with inversions among 197 Zambia genomes (Table S2; see Materials and Methods). We then implemented a resampling method to test for significant inversion frequency changes between parents and offspring (while accounting for the variance observed in technical replicates; see Materials and Methods), in order to determine whether males carrying each inversion were more or less successful in reproducing than the average male. We also compared frequencies between embryos and aged adults that

454 eclosed either in the first or second half of the collection period, to investigate the relationship between inversions
455 and viability/longevity. We calculated p-values from the resampling tests to assess the significance of each
456 individual change in inversion frequency between relevant samples (Table S3). However, in accord with our
457 primary question regarding fitness tradeoffs, we primarily focus here on joint tests of two opposing frequency
458 changes, as further described below. We could therefore test the hypothesis that a given inversion had opposing
459 influences on survival and male reproduction, which would provide evidence consistent with our model of
460 balanced sexual antagonism, while also examining whether any such tradeoffs depend on maternal genetic
461 background.

462



463

464 [**Figure 8:** The layout and potential expectations of the laboratory evolution experiment. We cross outbred males
465 from a high-inversion population to inbred, non-inverted females from a specific inbred line, and collect DNA
466 samples from the fathers, embryo offspring, and aged adult offspring (left). We hypothesize from our antagonistic
467 pleiotropy model that the inversions will experience opposing selection between fathers and embryos versus
468 between embryos and aged adults (middle). To test the significance of observed frequency changes, we designed a
469 resampling model to represent the sources of neutral variation expected in the experiment (right).]

470 When we initially examined the joint effects of male reproduction and survival, we found that in a majority of
471 cases, a given inversion increased in frequency in adult offspring compared to the null expectation based on the
472 parental male pool (in 13 out of 16 inversion-line trials; binomial $P = 0.02$; Figure 9). Given that inversion
473 homozygotes are not generated by our crossing design, these results are likely to indicate that inversion
474 heterozygotes often had higher fitness than standard homozygotes (across male reproduction and survival jointly).
475 We note that our standard homozygotes are not identical-by-descent but are instead heterozygous for unique
476 standard-arrangement chromosomes from two independent Zambia strains, and that long genomic tracts of
477 identity-by-descent between Zambia strains are quite rare in this large, ancestral-range population (Lack et al.
478 2016a). Hence, we do not expect that inversion heterozygosity is buffering against deleterious effects of
479 inbreeding. Instead, cases where inversions are associated with fitness advantages suggest either a simple
480 directional advantage of inversion-associated alleles for a given fitness trait, or else an advantage for heterozygous
481 inversion/standard genotypes in particular. In the former case, the inversions may still be under balancing
482 selection in the wild, if the equilibrium frequency of the inversion was shifted due to lab conditions such that one
483 karyotype was always favored in the lab environment tested. This scenario would mirror previous findings in which
484 inversions found at intermediate frequencies in natural *D. melanogaster* populations (Inoue 1979), as well as
485 inversions from seaweed flies known to be under balancing selection in the wild (Mérot et al. 2020), were both
486 inferred to be under purely directional selection in the lab. We might still expect some difference in benefit across
487 the life history of a single generation if there is a balanced tradeoff in some alternate environment or at a different
488 frequency, consistent with the differences observed in our populations.

489 When we separated the effects of male reproduction (based on father to embryo changes) and survival (embryos
490 versus adults), we found that the effects of inversions on survival were generally consistent across maternal
491 genotypes. Three inversions were associated with increased survival: *In(3R)K* was in all four crosses, while *In(2L)t*
492 and *In(2R)NS* each showed elevated survival with all maternal strains except ZI418N (Figure 9A). In contrast,
493 *In(3L)Ok* was associated with lower survival in each cross (Figure 9A). With regard to male reproduction, the
494 trajectory of inversion frequencies from fathers to embryos to offspring was less consistent between different
495 crosses (Figure 9A), implying that differences in male reproductive success were dependent on the maternal

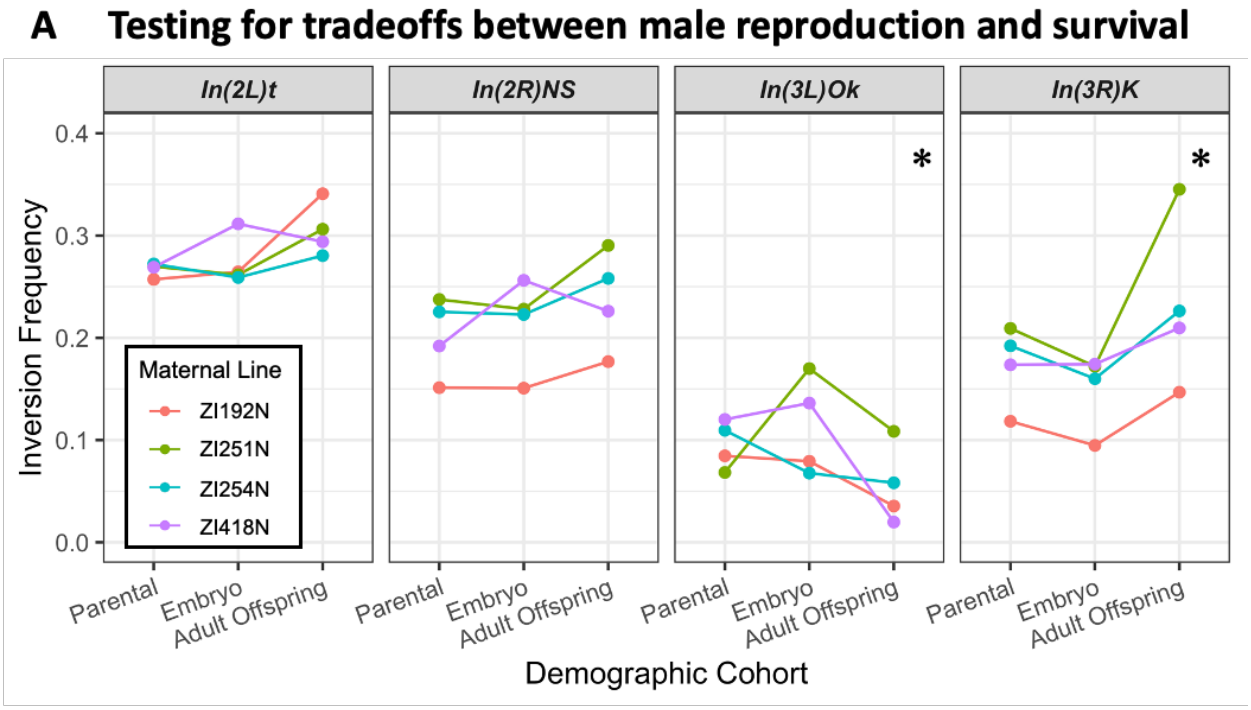
genotype, which is consistent with past findings in *D. melanogaster* (Reinhart et al. 2015). Despite such variability, each of the four inversions had multiple frequency changes that departed strongly from null expectations (Figure 9A; Table S3), as might be expected if selection was present, but varied in strength and direction between crosses and life stages.

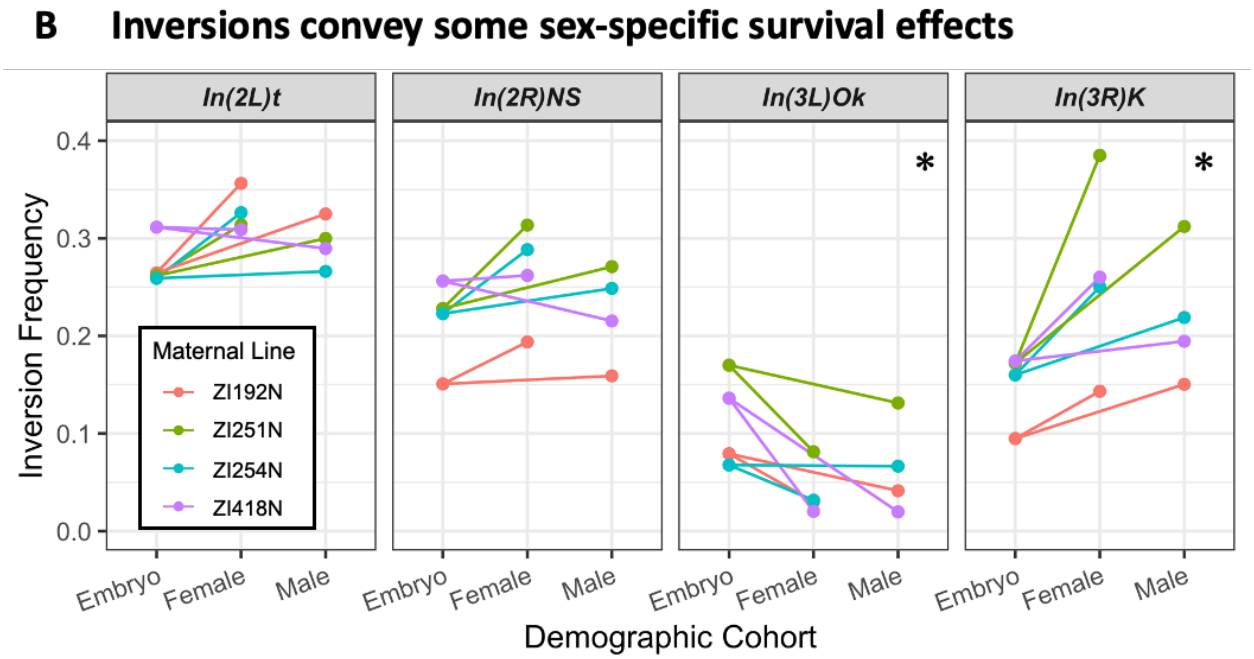
To test our central hypothesis of a tradeoff between male reproductive success and cumulative survival during development and adult aging, we evaluated the significance of each change in inversion frequency between the paternal and embryo cohorts, alongside the change in inversion frequency between the embryo and combined adult offspring cohorts. Among these results, *In(3R)K* had particularly consistent changes in frequency across replicate lines, with a decrease between parents and embryos, and then an increase between embryos and aged offspring (Figure 9A). When we assessed the likelihood of observing contrasting frequency shifts between life stages of this magnitude, integrating across the four maternal crosses and accounting for multiple test correction, we found that the *In(3R)K* pattern was significantly non-random ($p = 2.70 \times 10^{-3}$; Table S4; see Materials and Methods). The other inversion that gave reversed frequency shifts from cross-averaged data was *In(3L)Ok*, which unlike *In(3R)K*, was associated with (variably) higher male reproductive success and (consistently) lower survival. The frequency reversal observed for *In(3L)Ok* was also found to be statistically significant ($p = 2.97 \times 10^{-7}$).

We also tested for frequency differences between the different sexes of adult offspring and between the early-eclosing and late-eclosing cohorts of adult offspring. All four inversions displayed fairly consistent differences in the production of female versus male offspring (Figure 9B), suggesting that each of them have differential effects on sex-specific survival. Strikingly, *In(3L)Ok* and *In(3R)K* each displayed sex survival differences in alignment with their antagonistic effects implied above: *In(3L)Ok* favored male survival more-so than female survival ($p = 0.0450$), whereas *In(3R)K* favored the survival of females over males ($p = 4.61 \times 10^{-3}$; Table S4; see Materials and Methods). The other two inversions, *In(2L)t* and *In(2R)NS*, each consistently favored female over male survival in all four maternal crosses, although their initial unidirectional cross-combined *p* values (0.0260 and 0.0126) were not significant after multiple test correction ($p = 0.282$ and 0.137). In contrast, we found no consistent effects of inversions on eclosion time (Figure S3). Overall, we conclude that *D. melanogaster* inversions have distinct and

521 often context-dependent influences on male reproduction and on survival, with some evidence for antagonistic

522 tradeoffs of the sort proposed in our model.





523

524 [**Figure 9**: Some *Drosophila* inversions show evidence for antagonistic tradeoffs and sex-specific survival. (A)
525 Testing for antagonistic fitness effects on reproduction and survival. Each inversion's frequency in parents, embryo
526 offspring, and adult offspring are shown for each maternal inbred line. Parental frequency is taken as the paternal
527 frequency divided by two to account for the inversion-free inbred mothers. Adult offspring frequencies are taken
528 from the sum of estimated allele counts across all adult offspring cohorts. * indicates p-value < 0.05 for evidence of
529 significant reversed frequency change from parental to embryo and from embryo to offspring, combined across all
530 four maternal line crosses and corrected for multiple tests, when compared to neutral simulated experiments
531 (details in Materials and Methods). (B) Testing for evidence of sex-specific survival effects. The changes in inversion
532 frequencies between embryos and both female and male adult offspring are illustrated. * indicates p-value < 0.05
533 for significant differences in female versus male inversion frequency among adult offspring, combined across all
534 four maternal crosses and corrected for multiple tests.]

535 3 Discussion

536 Here, we have introduced a new conceptual model in which (1) sexually antagonistic variation is present and
537 subject to balancing selection that maintains extreme genotypes favoring either male reproduction or survival, and
538 (2) inversions are predicted to facilitate the aggregation of progressively more antagonistically differentiated
539 haplotypes. We have tested this model using simulations, confirming the predicted efficacy of balancing selection
540 and demonstrating that inversions indeed facilitate the construction of haplotypes containing multiple antagonistic
541 variants. Finally, we have deployed *Drosophila* lab experiments to uncover some evidence for inversion-associated
542 tradeoffs between reproduction and survival, depending on the specific inversion and strains tested.

543 3.1 Inversions in *D. melanogaster*

544 We have presented results from a single generation laboratory evolution experiment, in which we crossed outbred
545 male *D. melanogaster* carrying inversions *In(2L)t*, *In(2R)NS*, *In(3L)Ok*, and *In(3R)K* to four separate inbred female
546 lines and tracked the frequency of the inversions in the initial males, embryonic offspring, and aged adults. For

547 most inversions, the strength and direction of the tradeoff varied notably between crosses to different inbred
548 maternal lines, mostly due to maternally-dependent variation in male reproductive-success. However, greater
549 consistency was observed for *In(3R)K*, which was associated with lesser male reproductive success but greater
550 survival. A significant tradeoff was also inferred for *In(3L)Ok*, which favored male reproduction (albeit variably)
551 over survival. These results provide significant but measured evidence for the presence of sexually antagonistic
552 autosomal inversions in this model species, while emphasizing the importance of genetic background.

553 As indicated in the Introduction, models like ours in which inversion frequencies are influenced by balancing
554 selection on pleiotropic tradeoffs may help explain multiple aspects of observed *D. melanogaster* inversion
555 frequencies. This class of models inherently accounts for the intermediate maximal frequency of most *D.*
556 *melanogaster* inversions in natural populations. To the extent that inversions favor male reproduction, this could
557 also help account for the predominantly autosomal locations of common inversions in this species, given that
558 male-specific fitness effects have a weaker influence on the frequencies of X-linked variants since the X
559 chromosome spends only one third of its time in males.

560 This model could also help explain the observed geographic variability of inversion frequencies, as a change in
561 environment may change the fitness value of either pleiotropic effect of the alleles. If a population experiences a
562 novel harsh environment with low survivability, then the population density may decrease, and the number of
563 encountered competing males may decrease as well, reducing the relative fitness advantage of enhanced male
564 display. If there is a genetic tradeoff between mating display and viability, the equilibrium frequency of an
565 antagonistic inversion that favors the genetic extreme of display should be lower. Additionally, in hostile
566 environments, the survival cost of display-favoring variants could be increased, which could further shift their
567 equilibrium frequencies downward or render them not worth carrying at any frequency. These predictions could
568 contribute to the observed negative relationships between some inversion frequencies and latitude or altitude
569 (Kapun et al. 2016; Pool et al. 2017). As a specific example, it is notable that in a high altitude Ethiopian
570 environment with year-round cool weather, which hosts some of this species' most pronounced phenotypic
571 evolution (Bastide et al. 2014; Lack et al. 2016b), inversions are virtually absent - occurring at the lowest

572 frequencies of any analyzed population (Sprengelmeyer et al. 2020). Consistent with such reasoning, a
573 disadvantage of some inversions in challenging thermal environments (both cold and hot) was supported by a
574 previous experimental evolution analysis (Kapun et al. 2014). However, our experimental results include evidence
575 that some inversions, such as *In(3R)K*, may have the opposite tradeoff - that the inverted arrangement is
576 associated with survival success instead of reproductive success. Notably, we have only tested survival and
577 reproduction in a benign laboratory environment, and not under challenging environments such as cold
578 temperatures. Other studies in *D. melanogaster*, including those on focused seasonal evolution, have found
579 evidence for important tradeoffs between reproduction and robustness to environmental challenges (*e.g.*
580 Behrman et al. 2015). Hence, it would clearly be desirable to study the effects of inversions on a wider range of
581 potential tradeoffs. For example, Said et al. (2018) suggested that inversions *In(2L)t* and *In(3R)K* might be
582 maintained by immunity-related tradeoffs, in light of an over-representation of immune-related genes among
583 differentially expressed transcripts between inverted and standard karyotypes.

584 In addition to a broader array of balanced tradeoffs that could modulate *D. melanogaster* inversion frequencies,
585 drift and other forms of selection may contribute as well. Although we emphasize above the potential interplay
586 between ecological adaptation and balanced tradeoffs, some inversions could be impacted by simpler forms of
587 directional selection. However, we must then invoke secondary explanations to account for their limited maximal
588 frequencies (see Introduction). Other forms of balancing selection may also come into play as well. It is clear that
589 some variation in this species is subject to temporally-varying selection, and some inversions have displayed
590 seasonal frequency differences (Kapun et al. 2016; Lange et al. 2022). As with spatial clines, temporal shifts could
591 reflect either simple directional selection or else environmentally-modulated tradeoffs in a model such as ours.
592 Spatial selective pressures may vary both between populations and within population scales.

593 As indicated in the Introduction, balancing selection on inversions could also be achieved by 'associative
594 overdominance' (Zhao and Charlesworth 2016; Gilbert et al. 2020), where two haplotypes are balanced by
595 recessive cost to both homozygotes. Inversions may be particularly susceptible to carrying recessive deleterious
596 alleles if population size and/or inversion frequency are low, since under these circumstances inversion

homozygotes are rarely generated and hence the crossover rate among inverted chromosomes is low, plus there are fewer inverted chromosomes to facilitate rare double recombination events with standard chromosomes. However, this dynamic is expected to decay with increased age and population size of the inversion, as mutation, gene conversion, or double crossover resolve the negative linkage between recessive deleterious alleles. Most common inversions in *D. melanogaster* are both relatively old and have large effective population sizes. Inversions including *In(2L)t*, *In(2R)NS*, and *In(3R)K* have breakpoints estimated to be several times older than the expansion of the species out of southern-central Africa (Corbett-Detig and Hartl 2012; Sprengelmeyer et al. 2020). Those three inversions, plus *In(3L)Ok*, for which inversion age has not been estimated, all maintain frequencies greater than 0.15 within very large populations (Sprengelmeyer et al. 2020), and so it is not clear that the persistence of recessive deleterious or lethal alleles in the inverted regions is expected to be so much higher than for standard arrangements that inversions should be maintained by associative overdominance. In agreement with these predictions, many wild-derived inbred strains of *D. melanogaster* are homozygous for inverted haplotypes (Lack et al. 2016a). Furthermore, as noted in the Results, even an observed lack of inversion homozygotes could reflect strong sexual antagonism as opposed to recessive deleterious variation. Nevertheless, further simulation and experimental testing regarding the potential influence of associative overdominance and other processes on inversion frequencies in *D. melanogaster* and other species would be desirable.

3.2 Generality of Balanced Pleiotropic Inversions

We have proposed that some polymorphic inversions in *D. melanogaster*, and in other species with analogous mating dynamics, may be maintained in part through balancing selection acting on a frequency-dependent tradeoff. While we explored the tradeoff between male reproduction and viability, we emphasize that any antagonistic pleiotropy involving at least one frequency-dependent trait has the potential to generate similar inversion polymorphism. Such antagonistic pleiotropy may be common in evolution, and might be useful in explaining the many examples of inversions associated with distinct phenotypes.

620 For the specific model explored here, of antagonistic pleiotropy between survival and display in a mate choice
621 system, the prevalence of the dynamic across species and populations is unclear. It seems plausible that a similar
622 model of antagonistic pleiotropy and balancing selection under mate choice underlies the balanced inversion
623 system in the European ruff *Philomachus pugnax*, where three inverted haplotypes on an autosome determine
624 aggressive, cooperative, and female-mimic male lekking morphs, albeit with a dominance relationship and
625 homozygous inviability for an inverted haplotype (Küpper et al. 2016). More generally, a number of factors
626 contribute to the potential prevalence of this dynamic. Mate competition and choice during reproduction is rather
627 common among animals, and this may mean sexually antagonistic inversions are plausibly also common. The
628 model depends on the distribution of fitness effects of new mutations and the availability of sexually antagonistic
629 variation, but such variation does seem prevalent (Zajitschek and Connallon 2018; Ruzicka et al. 2019). More likely
630 to limit the prevalence of such inversions are the meiotic costs experienced by inversion heterozygotes in some
631 systems (White 1973), whether sexually antagonistic variants are clustered in a region that would allow inversions
632 to increase linkage, and whether other mechanisms like sex-biased expression (Ingleby et al. 2015), gene
633 duplication (Connallon and Clark 2011), and linkage to the sex chromosome or sex-determining locus would
634 resolve the antagonism more frequently (though see following subsection on sex chromosome evolution).

635 Perhaps more importantly, there are a number of well-studied cases of inversion polymorphism that may follow
636 this dynamic of balancing selection between haplotypes demonstrating antagonistic pleiotropy, but which do not
637 involve sexual antagonism. *Papilio* butterfly populations harbor well-studied mimicry polymorphisms that
638 segregate between inverted segments and may often experience frequency-dependent selection (Clarke and
639 Sheppard 1960; Kunte et al. 2014; Poul et al. 2014). The snail *Cepaea nemoralis* harbors diverse and distinct shell
640 patterning haplotypes associated with inversions that remain balanced within single sampling locations (Cook
641 2013; Surmacki et al. 2013). Several inversions in different ant species segregate a single-queen colony strategy
642 from a cooperative multi-queen strategy within the same range (Wang et al. 2013; Libbrecht and Kronauer 2014).

643 Essentially, any population with antagonistic pleiotropy involving some form of negative frequency-dependent
644 balancing selection on one of the phenotypes might exhibit the same dynamic. The population would experience

similar divergent selection towards alternate phenotypic extremes, defining an emergent level of epistasis between alleles that trade off between the pleiotropically determined traits. This idea integrates with existing thought on the evolution of recombination and divergent selection on balanced systems. For example, Kopp and Hermisson (2006) have proposed a specific form of frequency dependent divergent selection (FDDS), involving competitive exclusion between similar genotypes for a trait otherwise under stabilizing selection, and this model was expected to result in single or few loci of large effect. Their model did not consider pleiotropy or linkage modifiers, but still had comparable dynamics in that selection in the FDDS regime favored small numbers of large effect loci, while the inversion haplotypes in the model presented here simplify the recombination architecture to approximate a single locus. This simplification of the genetic architecture can be further seen as related to the reduction principle in recombination modifier theory (Feldman et al. 1980; Altenberg et al. 2017). When a population experiences selection towards two (or more) divergent optima, under a selection regime that provides a balancing mechanism to maintain both, indirect selection will favor a reduction of the genetic architectures to prevent the generation of intermediate phenotypes. The likelihood of either model likely rests on the inherent polygenicity of the trait under selection, with simpler traits being more likely to resolve by single locus FDDS dynamics than by inversion association (*e.g.* Yassin et al. 2016).

3.3 Autosomal inversions and sex chromosome evolution

If balanced, sexually antagonistic, autosomal inversions occur frequently enough, they may also contribute to the evolution of sex chromosomes. Given an established inversion with significant sexual antagonism, the inverted haplotype that favors one sex still experiences an antagonistic cost of transmission to offspring of the opposite sex. Mutations that link the haplotype with the sex it benefits are therefore favored under selection, and this can occur either by fusing to an existing sex chromosome (Charlesworth and Charlesworth 1980), though this only fully resolves the antagonism for linkage to Y and W chromosomes, or by linkage to a new sex determining mutation (van Doorn and Kirkpatrick 2007). In particular, we discovered that strong inversion-linked antagonism could yield a scenario where essentially all highly-successful males were homozygous for a display-favoring karyotype, whereas reproducing females were overwhelmingly heterozygotes (Figure 5). This situation is similar to the ZW sex

670 chromosome system, suggesting that this particular pleiotropic trade-off may favor resolution by linkage to a
671 female sex determination factor. Further, simulations in which only females experienced antagonistic survival cost
672 usually generated populations with a chromosome essentially lethal in females, thus exclusively transmitted in
673 males similar to a Y chromosome (Figure 7). These simulations sometimes had three states that appeared stable at
674 20*N* generations, including Y-like, W-like, and shared intermediate arrangements. This three-arrangement stable
675 polymorphism seems unusual for a natural population, but might be interesting to explore in populations with
676 polymorphic sex determination.

677 There are often deleterious effects of chromosome fusion or novel sex determination (Pennell et al. 2015;
678 Saunders et al. 2019), which may represent a general obstacle to sex chromosome evolution. However, balanced
679 sexually antagonistic inversions may concentrate enough antagonism that the built-in benefit of resolving it may
680 sometimes overcome the fitness costs of evolving new sex chromosomes (Blackmon and Brandvain 2017). Hence,
681 antagonistic inversions might be more likely than other loci to be involved in the evolution of new sex
682 chromosomes.

683 A basic prediction of the above hypothesis is that at least one inversion difference between sex chromosomes
684 should already exist at the time of new sex chromosome formation, and genomic differentiation therefore begins
685 between inversion karyotypes on an autosome even before sex-specific transmission of the region. This contrasts
686 with current models of sex chromosome evolution in which inversion accumulation and genomic differentiation
687 accrue only after sex chromosome formation, due to advantages in suppressing recombination involving sexual
688 antagonistic variants already associated with a sex-determining locus (Wright et al. 2016). Additional genomic
689 studies of new sex chromosomes are needed to assess the potential role of sexually antagonistic inversions in sex
690 chromosome evolution.

691 Summary and Future Prospects

692 We have advanced a model in which inversion polymorphism is maintained by the frequency-dependent fitness
693 effects of a pleiotropic tradeoff associated with inversion-linked genetic variation. We have used sexual

antagonism associated with increased male reproduction versus survival as a specific motivating case, and we explore the predictions of this model using a novel simulation algorithm. We confirmed model predictions that some antagonistic variants can be maintained at an equilibrium frequency, and that inversions can allow multiple antagonistic variants to persist and form more strongly antagonistic haplotypes. We complemented our conceptual and computational modeling with laboratory experiments designed to detect fitness effects of four inversions common in an ancestral range population of *D. melanogaster*. We indeed found some evidence of tradeoffs between male reproduction and survival for these inversions, with two out of four inversions showing significant evidence for such a tradeoff, although we also observed a strong dependence of male reproductive success on female genetic background.

In light of the persistent mystery of how *D. melanogaster* inversion frequencies are determined, and how readily we detected tradeoffs involving male reproduction and survival, we suggest that further studies investigating the potential fitness tradeoffs of inversions in this model system are strongly warranted. While some such studies have previously been conducted, the applicability of our amplicon sequencing approach to estimate inversion frequencies in large pools of flies may improve the sensitivity of such experiments. Our understanding of the phenotypic impacts of *D. melanogaster* inversions would benefit from investigations of a wider range of fitness-related traits, including female fecundity, immunity, and performance in challenging environments such as cool temperatures and high population densities.

Clearly, it will also be important to test the generality of our model by estimating the fitness associations of inversions in a wider range of species. And particularly in the case of species with very recent changes sex chromosomes, it will be of great interest to test whether one or more inversions are already present between these new sex chromosomes, as predicted a model in which sex chromosome evolution involves the resolution of inversion-associated sexual antagonism. Such studies will advance our broader understanding of the roles of balancing selection, pleiotropic tradeoffs, and genome structure in shaping genetic variation and genomic evolution.

4 Materials and Methods

4.1 Simulations

We developed a new individual-level forward simulator ('SAIsim' for Sexually Antagonistic Inversion simulator) written in python 3.7 for the simulation of inversions and sexually antagonistic alleles at infinite loci, with uniform rates of mutation, crossing over, and gene conversion (per bp, per generation) over a number of independent chromosome arms. The dynamic we were interested in modeling requires direct accounting of pleiotropy between different components of fitness, crossing over and gene conversion within inversions (as well as the possibility of inversion fixation), and directly modeling male display and female choice, and no existing simulator provided this functionality. These simulations were used to establish the potential for the model to occur in a population generally, and not to compare against specific empirical observations. We have used literature estimates of mutation rate and conversion rate of Zambian *D. melanogaster* for these simulations (Comeron et al. 2012; Huang et al. 2016), but have no information on the joint distribution of effects of mutations on our model of survival and display in *D. melanogaster*. So, we sample from relatively broad but arbitrary combinations of mutation effects, only assuming that there is likely some degree of antagonistic pleiotropy naturally present in genomic variation.

In SAIsim, a population is instantiated as a python object, and populated with individuals (also python objects) based on provided (or initially empty) genomes. Each generation, as diagrammed in Figure 5, the population calculates a survival probability for each individual as the product of the survival values of each variant they carry, which range [0,1]. Male and female survival costs can each be included in a sex-specific manner, but only globally in the simulation, not per mutation. For each offspring in the next generation, a mother is selected randomly, weighted to survival values, with replacement. Then a set number of potential fathers (*i.e.* the uniformly specified encounter number) is randomly sampled for each offspring from the full population, weighted by their survival values. The males each have a display quality given as the sum of the reproductive values of their alleles, plus a normally distributed additional noise with standard deviation equal to one display quality unit. SAIsim allows the specification of mutation rates and the distribution of effect sizes of new mutations; our simulations use mutations

743 with uniformly distributed quality values in the range [0,1]. A noise parameter, normally distributed with a
744 standard deviation of 1, is also applied to each male display value, with the noise effect generated separately for
745 each interaction. Once the male display qualities are calculated based on genotype and noise, the mother of each
746 offspring chooses a father deterministically by the highest quality. As offspring are generated, crossover locations
747 are sampled, and resampled in cases where the resultant gamete would be aneuploid. Gene conversion is modeled
748 as a flat probability per locus, with each event independently affecting just one locus, rather than modeling a
749 conversion tract length. Inversions and new mutations are added to the resultant gamete, with inversions
750 resampled when overlapping an inversion present on the chromosome. The simulation is intended to represent *D.*
751 *melanogaster* life history and allows removal of recombination in males, though this also removes gene conversion
752 by default.

753 SAIsim's object-oriented design allows easy specification of a population model by a new user familiar with the
754 basics of python scripting. A population object can be parameterized and populated in a few lines of python script,
755 and then parameters can be changed between periods of simulation, allowing bottlenecks and demographic
756 changes as well as changes in mutational parameters between generations. Replicate SAIsim simulations were
757 performed via the UW-Madison Center For High Throughput Computing (CHTC), which manages cycle servers
758 using the HTCondor distributed computing project (Thain et al. 2005; Erlandson and Theisen 2018).

759 4.2 Experimental Populations and Sequencing

760 To act as a high-inversion source population for the potential fathers in our experiment, we combined 15 males
761 and 15 females from each of 10 inbred strains derived from wild flies collected in Zambia. These 10 strains were
762 chosen to collectively carry the following common inversions: *In(2L)t*, *In(2R)NS*, *In(3R)K*, *In(3R)P*. We then collected
763 at least 500 F2 males from this high-inversion population to use in each of our independent maternal line
764 experiments, and these males were expected to carry a wide range of inversion genotypes. Each batch of high-
765 inversion males was crossed to an equal number of virgin females from a single inbred Zambian strain (Figure 6).
766 Four such experiments were performed, each using one inversion-free maternal strain. Since no inversions could

767 be inherited through the mothers, inversion frequencies of successfully reproducing F2 males could be inferred
768 from their pooled offspring. Unless otherwise noted, all flies were kept in a lab space of 23°C with around a degree
769 of temperature fluctuation and without a controlled day/night cycle. All fly food used was prepared in batches of
770 4.5 L water, 500 mL cornmeal, 500 mL molasses, 200 mL yeast, 54 g agar, 20 mL propionic acid, and 45 mL tegosept
771 10% (in 95% ethanol). The four parental populations were each allowed to mate freely in clear plexiglass cages of
772 dimension 19.3 by 19.3 by 30.0 cm, with a loose mesh covering on one end for access by hand, twisted and clipped
773 shut with a binder clip. The parents were given either fly food bottles or grape agar in 7.9 cm petri dishes as food
774 or laying substrate on alternating days over two weeks, after which all surviving male parents were counted and
775 frozen. The grape agar was prepared in batches of 500 mL from FlyStuff.com grape agar powder, and wet Red Star
776 yeast was brushed over the agar plates before use to encourage laying. Embryos were collected from the agar,
777 counted, and frozen for sequencing. Embryos laid in the bottles were allowed to hatch and develop into adults.
778 These adult offspring were separated by sex and by the time after laying at which they eclosed. In our husbandry
779 conditions, the earliest eclosion was 11 days after laying, but this was rare and a first eclosion at 12 days was more
780 common among the bottles. Most flies took several days longer to develop, and we considered flies collected in
781 the first 6 days of a 10 day collection window to be the early-eclosing pool, in order to split the pool sample sizes
782 closer to evenly. The collected adult flies were then aged by sequentially transferring them between bottles
783 weekly for approximately 2.5 months, and then the adult offspring were sexed, counted, and collected. A 10-week
784 aging window was chosen based on the time taken for a control set of four bottles of flies from the paternal
785 outbred population to reach 80% mortality. All adult and embryo samples were frozen at -80°C before DNA
786 extraction. This sampling scheme was chosen to identify inversion frequencies in the set of potential parents, in
787 embryonic offspring, and in older adults, and so to assess potentially selected changes in inversion frequency
788 across male reproduction or in egg to adult viability plus longevity.

789 DNA for each replicate age group was extracted by a phenol-chloroform extraction protocol used previously in
790 preparing DNA for the Drosophila Genome Nexus and *Drosophila* Population Genomics Project. The protocol is
791 similar to protocol 'BPC' used in DPGP phase 1 (Langley et al. 2012), except with the addition of proteinase-K
792 during homogenization of adult flies, and generally based on the common quick preparation protocol presented in

793 *Drosophila Protocols* (Huang et al. 2009). Inversion genotyping was performed by amplicon sequencing, where
794 amplicons associated with each inversion were designed to target at least one single nucleotide polymorphism in
795 the inversion interior that is fixed for alternate alleles between the inversion karyotypes, while still sharing
796 conserved primer regions across karyotypes to prevent primer bias. Genetic variation in these inversions was
797 assessed using previously sequenced and inversion-called haploid genomes from the Zambian *D. melanogaster*
798 population, available from the *Drosophila* Genome Nexus (Lack et al. 2016a). The amplicon region and primer
799 selection was performed with a custom script. The script calls Primer3 version 2.4.0 for primer candidate
800 generation (Koressaar and Remm 2007; Untergasser et al. 2012; Koressaar et al. 2018). Primers thus selected were
801 ordered from IDT as oligos ligated to Illumina stubby adaptors [Table S1]. Illumina sequencing libraries were then
802 prepared for each sample following the Illumina metagenomic amplicon sequencing protocol (Illumina 2013), with
803 50 ng/uL diluted DNA samples. and sequenced 2x150 on an Illumina NovaSeq to at least a depth of 20000 reads.
804 Depth was selected to ensure high likelihood of detecting a 0.025 change in inversion frequency from an initial
805 frequency of 0.10, based on simulated resampling of the collected flies, DNA extraction, and sequencing.

806 4.3 Sequence Analysis and Statistics

807 Sequence preparation and analysis were performed using custom pipeline scripts. Read trimming, alignment, and
808 QC were performed using bwa version 0.7.17-r1188 (Li 2013), GATK version 3.4-46 (Van der Auwera and O'Connor
809 2020), and Picard version 1.79 (Broad Institute 2019). Reads were trimmed at the ends to remove segments with
810 base quality less than 20, and read pairs were filtered to retain only those pairs where both reads aligned with
811 mapping quality >20. SNP identity at the sites of interest was called from those remaining reads with base
812 quality >20 at the SNP of interest. Due to the presence of chimeric reads in the amplicon library sequencing, we
813 chose to use a single discriminant SNP to determine each inversion's frequency in the read pool, instead of
814 incorporating haplotype or additional SNP information.

815 To assess the significance of the difference in inversion frequency between two sampled life stages in the same
816 experiment, we first calculated a chi-square test statistic from the two-by-two table of inverted and non-inverted

817 allele counts at two life stages. Allele counts were taken by multiplying the inversion-specific SNP frequency in the
818 sequencing reads by the allele sample size. We then generated an approximate distribution of the chi-square
819 statistic when selection is absent (our null hypothesis), using a resampling approach (Figure 6). Comparing the
820 observed statistic to our modeled neutral distribution of test-statistic values allowed us to identify significance
821 thresholds.

822 To generate the neutral distribution of chi-square test statistics, we took the observed paternal inversion
823 frequencies and then sampled new paternal and offspring read sets to represent a single neutral sample. Ideally,
824 we could sequence from the pool of all fathers in the experiment, but up to 15% of the paternal population died
825 before collection. So, in generating a new paternal read set, we first modeled down-sampling of the paternal allele
826 sample from the initial census size to the collected allele size as a hypergeometric sampling. Then we used a
827 binomial sampling to account for the variation introduced by the extraction and library preparation. The extraction
828 and library preparation appeared to introduce variation independent of allele sample size, possibly due to
829 variation introduced during PCR or sample homogenization. This inference was based on analysis of data from
830 control library preparations (Table S5) and sequencing done in parallel with our experiment from sample fly pools
831 with known inversion frequencies. These control samples included an average of 6 technical replicates (separate
832 library prep and sequencing replicates from the same flies) from four different expected frequencies for *In(2L)t,* as
833 well as one extraction each for *In(2R)NS* and *In(3R)K*. Due to genotyping and husbandry difficulties we did not add
834 a control set for *In(3L)Ok*. Based on these technical replicates, we estimated that taking a binomial sampling of size
835 316 from the sample allele frequency reflected a maximum likelihood model for the sample-size-independent
836 variation introduced by the library prep and sequencing process. From the preliminary resampled paternal
837 inversion frequency that now accounted for the library variation, we then took a binomial sample of size equal to
838 the read count to account for sequencing variation, thus yielding resampled paternal read counts that account for
839 individual sample size, library preparation, and depth of coverage. For the new offspring read sets, we instead used
840 a binomial sampling of half the offspring alleles from the fully resampled paternal allele frequency for each
841 separate offspring cohort, since the homozygous mothers all contributed non-inverted alleles. Finally, each
842 offspring allele sample was resampled using the same size 316 binomial sampling to account for the variation

843 introduced during this library's preparation, and binomial sampled again with the read count size to account for
844 sampling reads from the library, thus yielding resampled offspring read counts that accounted for the same three
845 factors as for paternal counts.

846 For a pairwise comparison of inversion frequency between two samples, we calculated the same allele count chi-
847 squared test statistic as described above for the empirical data for each resampled set of allele counts, generating
848 the full distribution of neutral chi-squared test statistics from which we could identify extreme value cutoffs. We
849 then obtained a p-value from the proportion of resampled replicates in which the chi-square statistic was greater
850 than or equal to the empirical value. If the test was directional, we took the proportion of resampled replicates
851 with both a chi-square statistic greater than or equal to the empirical value and a frequency difference in the
852 expected direction.

853 In order to identify a frequency change reversal between life history stages that could reflect a fitness tradeoff, we
854 tested for the joint significance of two opposing frequency changes using three cohorts. A tradeoff would require a
855 frequency change in one direction between paternal and embryo samples, followed by a frequency change in the
856 other direction between embryo and adult offspring samples. These changes both depend on the shared
857 embryonic cohort in the test, and so are not independent. Therefore, we evaluated the two inversion frequency
858 changes jointly from a set of resampled neutral data sets in which the three cohorts arose from the same
859 resampling process. Here we considered how likely it was that a sample of the three cohorts from the associated
860 estimated neutral model had chi-square values for both the comparison of the fathers to embryos and the
861 embryos to combined adults that were equivalent or more extreme in the test direction than the observed data.
862 For example, if we were testing the significance of an observed paternal-embryo increase and then embryo-adult
863 decrease in frequency, our p-value would be defined by the proportion of resampled replicates in which the
864 frequency changes were both in these same directions, and their chi-square values were both more extreme than
865 the empirical values.

866 To assess the significance of inversion frequency changes considered across all four maternal line crosses at once,
867 we used Fisher's combined probability test. We applied this to both particular three-cohort frequency change

reversals and to the set of two-cohort frequency changes. The inversion trajectory is independent between maternal crosses, but the differences between each cross in maternal line and initial paternal genetic variation make the replicates difficult to compare in a more structured way, so we felt this was an appropriate test for combined significance. In assessing our tradeoff hypothesis, we applied this test only for inversions where it was well-motivated; *i.e.* where the averaged frequency change across maternal crosses was in opposite directions between paternal/embryo and embryo/adult comparisons.

To account for multiple hypothesis testing in our cohort comparisons, we used a Benjamini-Yekutieli false discovery rate correction for significance. We applied this correction across the set of Fisher's method combined p-values we generated to assess the relevant frequency change reversal hypotheses. So, this corrected for the multiple hypotheses of different tradeoff directions and different inversions involved, from p-values that already combined evidence from different maternal line crosses. Benjamini-Yekutieli was chosen due to its robustness to the unclear dependence relationships between some of the tests involved. For example, multiple tests of frequency changes from a single cohort (*e.g.* an embryo sample compared against both paternal and aged adult samples) are likely to be positively correlated.

Data Availability

Sequencing reads have been uploaded to the NIH Short Read Archive under project XXXXXX. The simulation program and other analysis scripts can be found at https://github.com/csmcal/.

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