

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

Reviewed Preprint

Published from the original
preprint after peer review
and assessment by eLife.

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Reviewed preprint version 1

December 28, 2023 (this version)

Sent for peer review

October 25, 2023

Posted to preprint server

October 4, 2023

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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 develop and apply a forward simulator to examine these dynamics under a tradeoff between survival and male display. These simulations indeed generate inversion-associated haplotypes with opposing sex-specific fitness effects. Antagonism strengthens with time, and can ultimately yield karyotypes at surprisingly predictable frequencies, with striking genotype frequency differences between sexes and between developmental stages. To test whether this model may contribute to well-studied yet enigmatic inversion polymorphisms in *Drosophila melanogaster*, we track 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.

eLife assessment

This **valuable** study introduces a new simulation model to explain the wide-spread occurrence of genetic inversions in fruit-fly genomes, based on sexually antagonistic alleles and a trade-off between male reproduction and survival. The evidence supporting the conclusions is currently **incomplete**, but it might be possible to address this with additional simulations and experiments as well as more rigorous analysis of the model and the data. The work will be of interest to population geneticists beyond the fruit-fly system.

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

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

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

Given the relevance of sexual selection and sexual antagonism to *Drosophila* evolution, we propose that polymorphic *D. melanogaster* inversion and standard arrangements may help maintain haplotypes of alleles that 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 selection will generate linkage disequilibrium between sets of associated alleles that together strongly favor either display or survival, and selection will favor the association of these haplotypes with alternate inversion karyotypes to reduce the recombination load experienced in heterozygous parents carrying one haplotype each from the extreme survival- and display-focused classes. We further predict that in a population that is close to the balanced equilibrium state, the population of adults will contain a higher frequency of survival-focused haplotypes than the population of zygotes, but the display-focused haplotypes will increase in frequency in zygotes relative to adults.

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

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

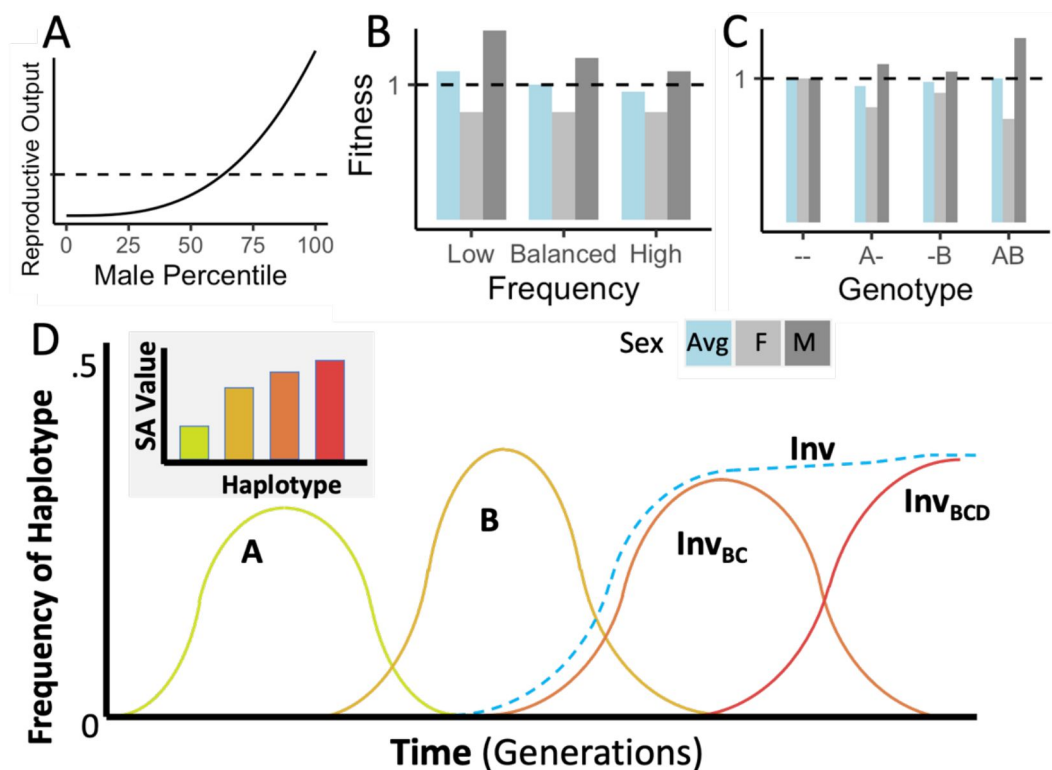


Figure 1

Conceptual representations of the proposed model of inversion-associated balanced sexual antagonism.

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

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.

2.2 Simulation Results

We investigated the predictions of the above model of inversion-associated balanced sexual antagonism using a purpose-built forward “Sexually Antagonistic Inversion simulator” (SAIsim; see Materials and Methods). This simulation program incorporates mutation of new nucleotide and inversion polymorphisms, crossing over and gene conversion (including between chromosomes heterozygous for inversions), drift, and sexually antagonistic selection (Materials and Methods). SAIsim allowed us to model the influence of polymorphic inversions on recombination, as well as the changes in linkage between sites entailed by the mutation and fixation of an inversion. It explicitly models mate selection, in that each female randomly selected to reproduce is assumed to encounter a number of randomly selected males, and the display values of each male are assigned based on the sum of genetic and environmental effects (with genetic effects based on genotypes at antagonistic loci), and the male with the highest display value then succeeds in this mating (**Figure 2**). We found it easiest to write a new simulator as no extant simulator accurately managed mutation and recombination of multiple inversion polymorphisms, as well as an explicit mate competition in generating offspring.

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

To test the effects of association with an inversion, we initially ran two sets of simulations, considering two antagonistic variants found to undergo balancing selection separately. First, we spaced the two antagonistic loci at a varying genomic distances, in the absence of an inversion (**Figure 4**). In each case, the stronger antagonistic variant (modelled at fixed position 0.225M) persists, and so we focus on whether the weaker variant (at varying 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.

Simulation Loop:

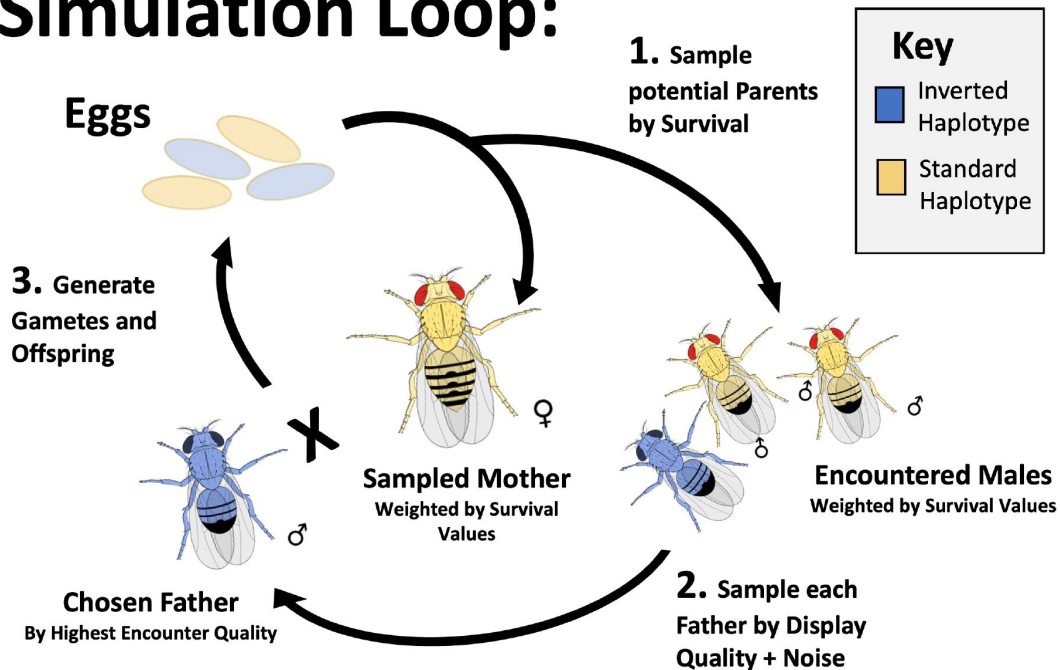


Figure 2

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

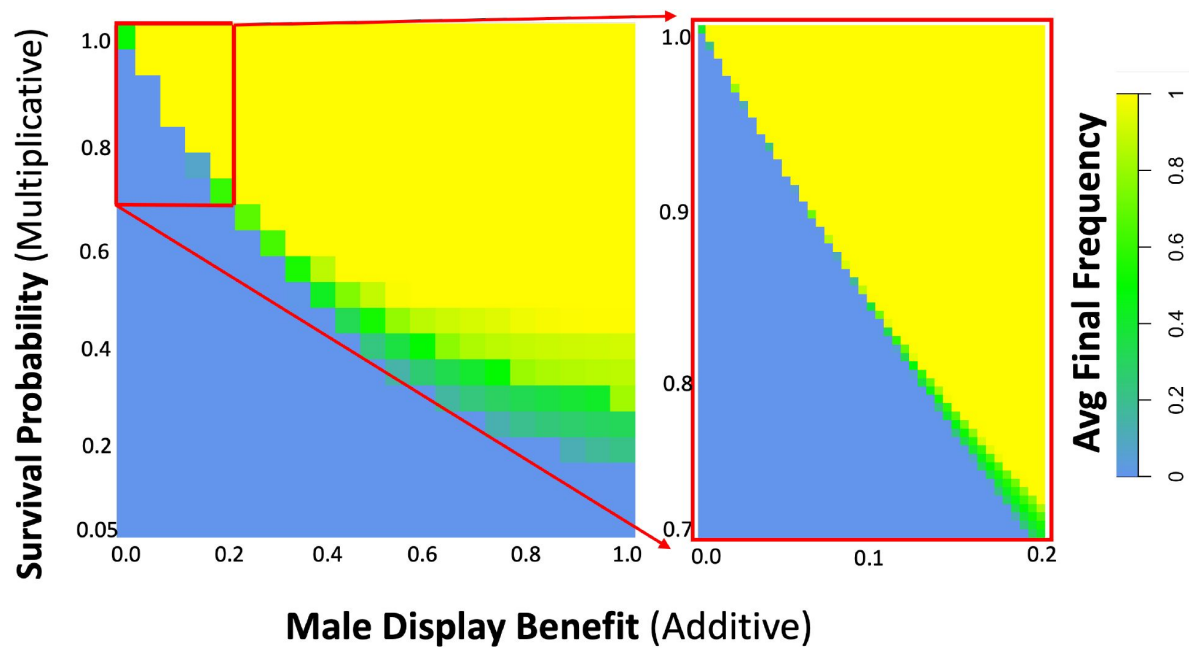


Figure 3

Simulations show that a balanced equilibrium frequency exists for certain sexually antagonistic variants. The average final frequency of a single mutation with given effects on survival proportion (of both sexes) and male reproductive quality score is shown, with green indicating intermediate balanced frequencies. These simulations involve a single antagonistic variant with initial frequency 0.5, in a population of 1,000 individuals simulated for 20,000 generations, with further details as indicated in the Materials and Methods. The left panel depicts trait values at 0.05 increments, whereas the insert presents a finer-scale set of simulations, every 0.005 trait units.

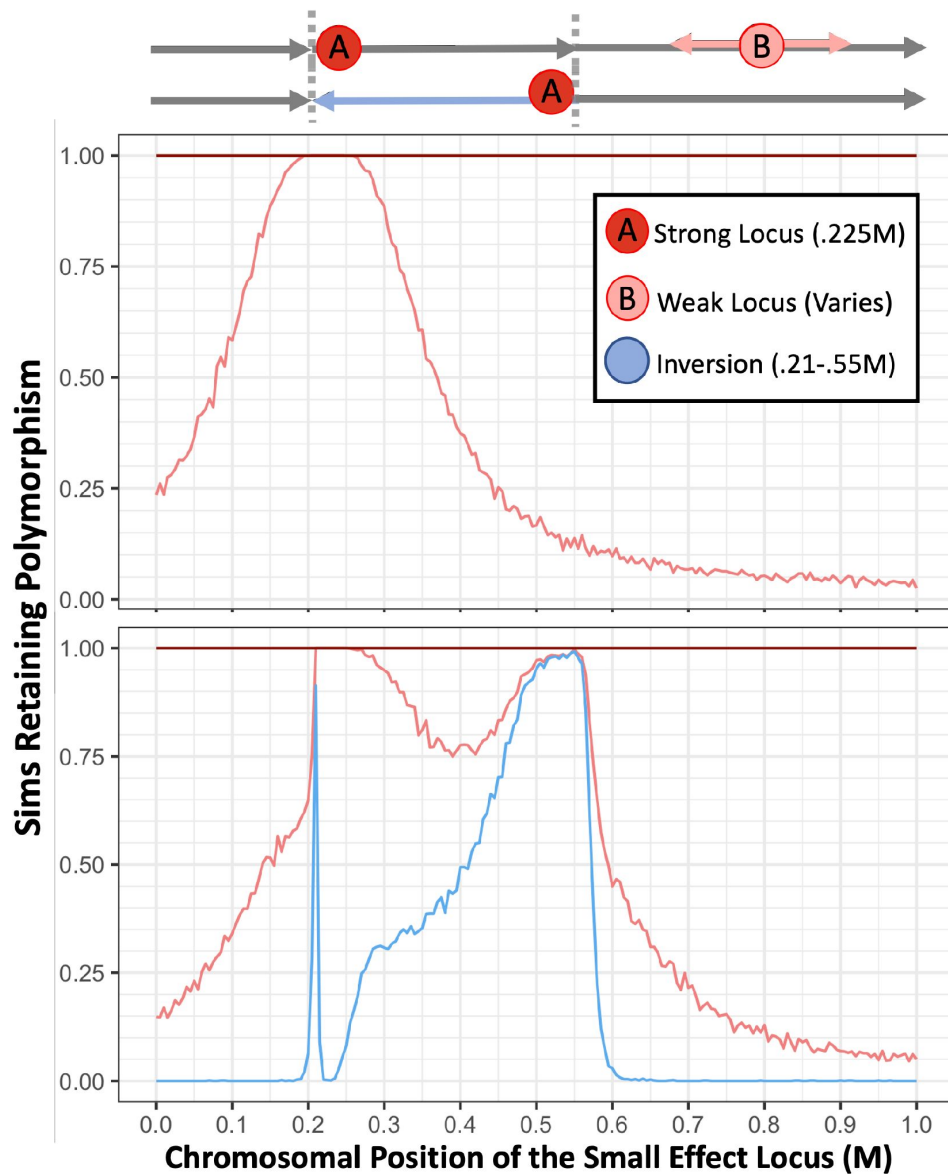


Figure 4

Simulated inversions persist as polymorphisms when linked to sexually antagonistic, pleiotropic variants. 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 20N generations in each parameter combination.

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 increase the crossover rate between the two antagonistic variants, and thus reduce the probability of of the weaker variant surviving by retaining linkage to the stronger one.

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

The simulations that successfully maintained balanced antagonistic chromosomes also had an interesting distribution of allele and genotype frequencies of the balanced haplotypes at equilibrium. Without any selection, the inversion frequency follows a pattern characteristic of the expected neutral site frequency spectrum (**Figure S2**). However, in parameter spaces where simulations with shared survival costs between sexes maintained polymorphism under balancing selection, inversion frequency distributions were tightly clustered around an intermediate frequency (**Figure S2**, 6A). Strikingly, the frequencies of alternate haplotypes consistently clustered around 0.25 and 0.75 among the zygote population. We found that this outcome was related to the genotype frequencies of reproducing adults. These simulations evolve toward strong antagonism, and it is predominantly males homozygous for the display-favoring haplotype (whether that is inverted or standard) who succeed in reproducing, and these males therefore contribute one copy of the display-favoring haplotype to each of their male and female offspring. However, this haplotype's significant cost to viability and longevity would select for the heterozygous females

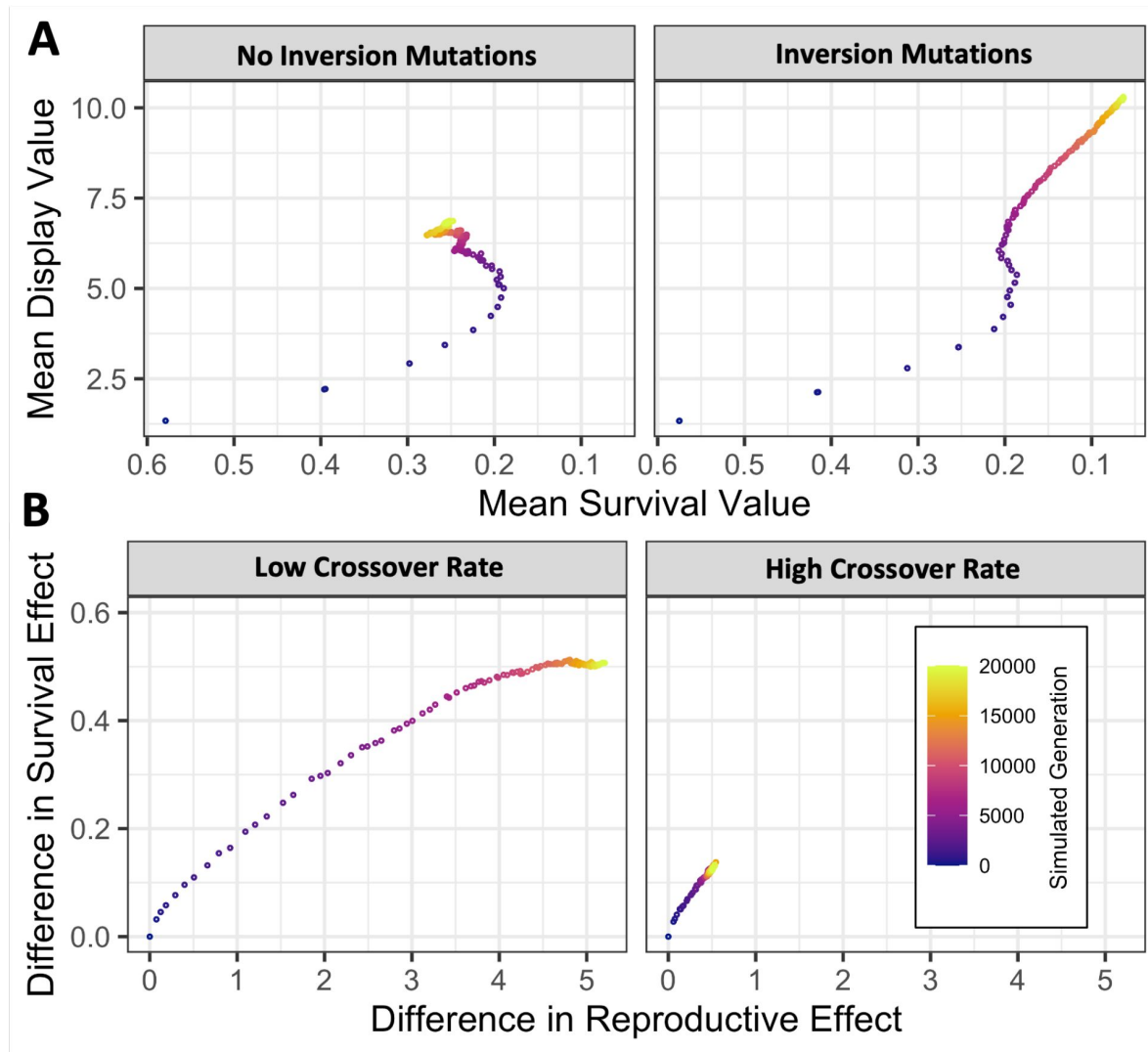


Figure 5

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

over the display-favoring homozygotes. Given that successful parents would predominantly be heterozygous females and display-homozygous males, three quarters of transmitted chromosomes have the display-favoring haplotype and one quarter does not, yielding the 0.25 and 0.75 inversion frequencies seen in simulations.

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

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

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

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

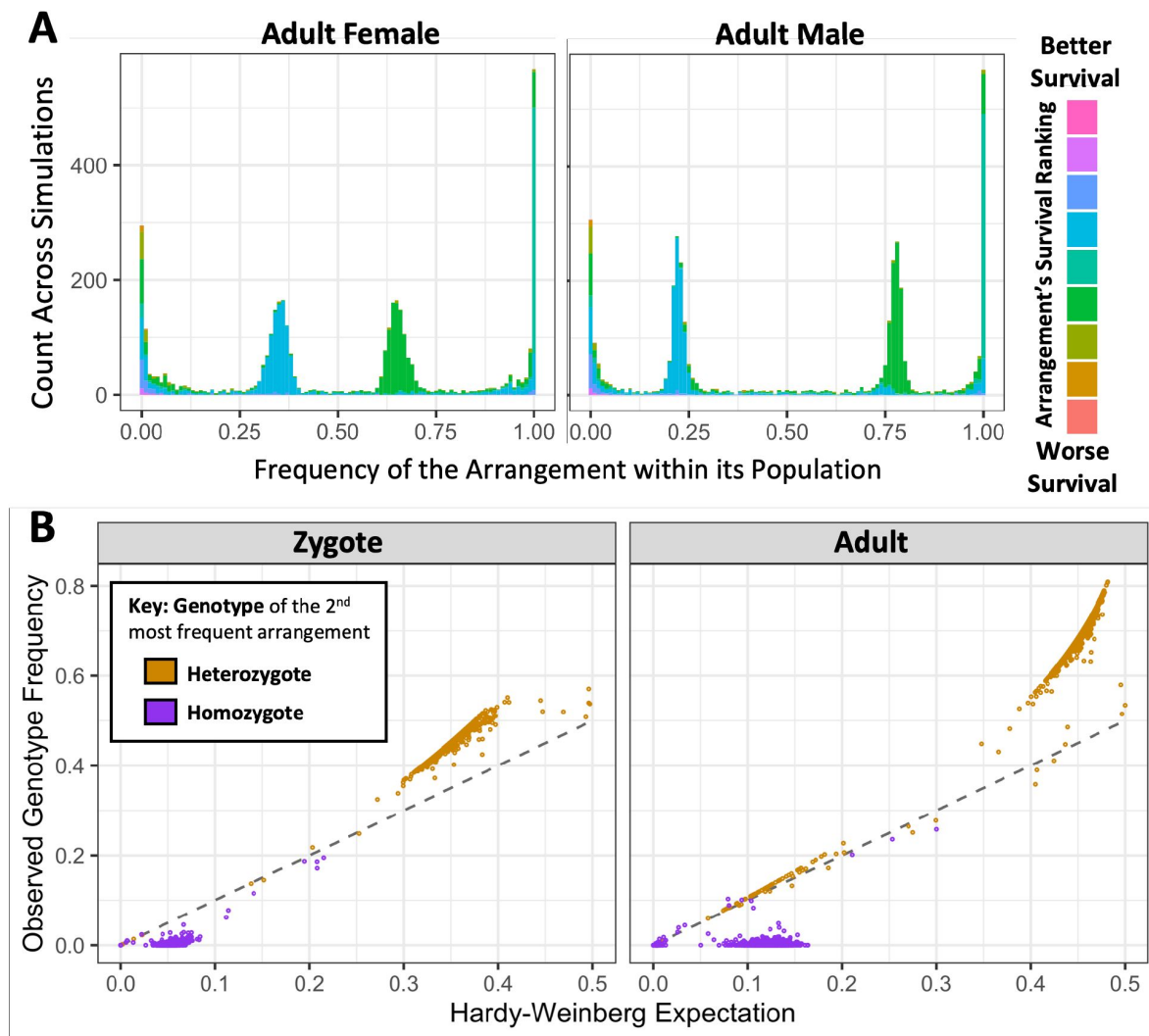


Figure 6

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

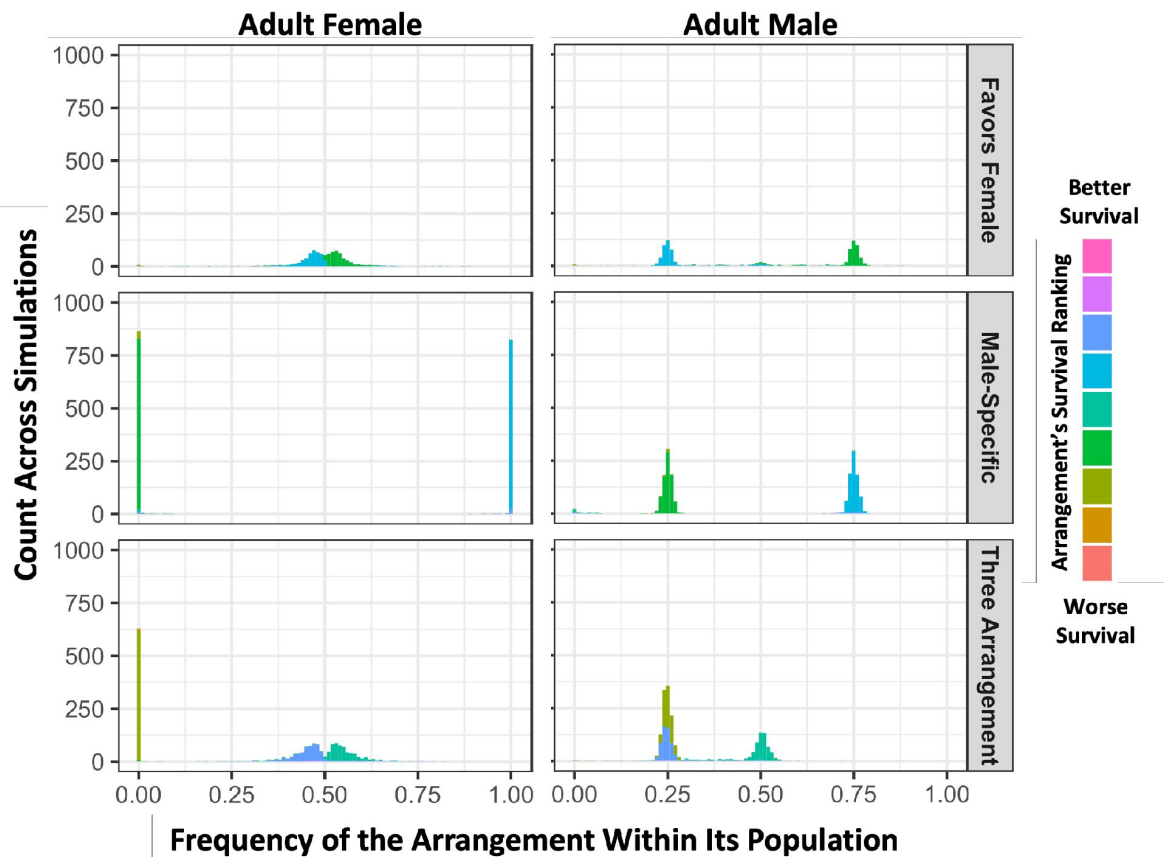


Figure 7

In simulations with female-limited survival costs, three distinct outcomes are observed involving either two or three balanced haplotypes. From simulations with randomly occurring antagonistic and inversion mutations, histograms of inversion frequencies across simulations for surviving females (left) and males (right) are partitioned into three categories based on sex-specific haplotype frequency outcomes. The “Favors Female” category (top) includes simulations in which there are exactly two arrangements with frequencies between 0.1 and 0.9 in both sexes (20.0% of replicates). The “Male-Specific” category (middle) includes simulations in which there 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.

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

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

When we separated the effects of male reproduction (based on father to embryo changes) and survival (embryos versus adults), we found that the effects of inversions on survival were generally consistent across maternal genotypes. Three inversions were associated with increased survival: *In(3R)K* was in all four crosses, while *In(2L)t* and *In(2R)NS* each showed elevated survival with all maternal strains except Z1418N (**Figure 9A**). In contrast, *In(3L)Ok* was associated with lower survival in each cross (**Figure 9A**). With regard to male reproduction, the trajectory of inversion frequencies from fathers to embryos to offspring was less consistent between different 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.

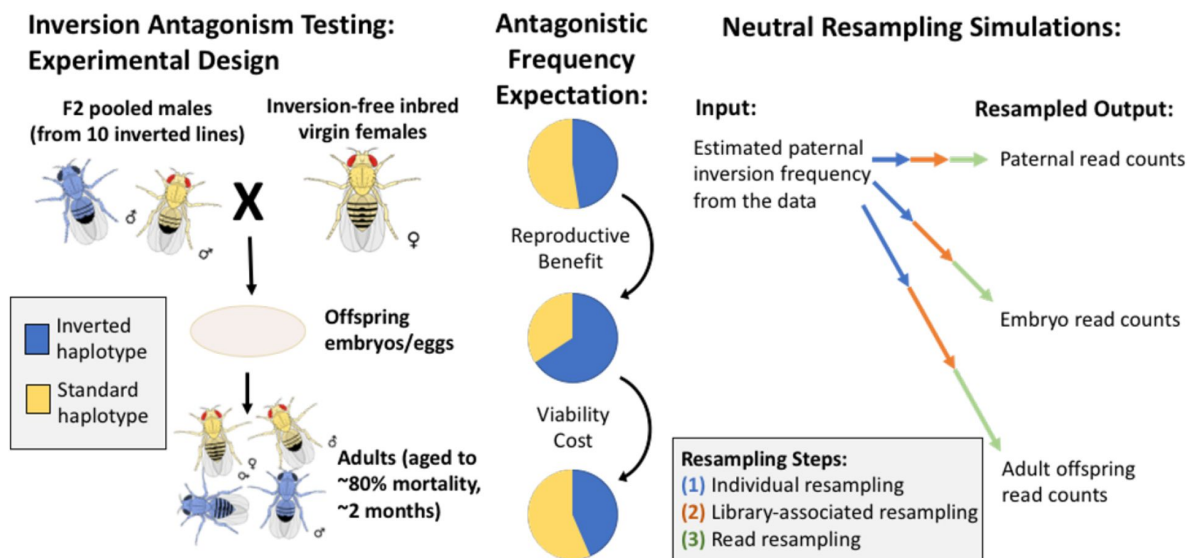
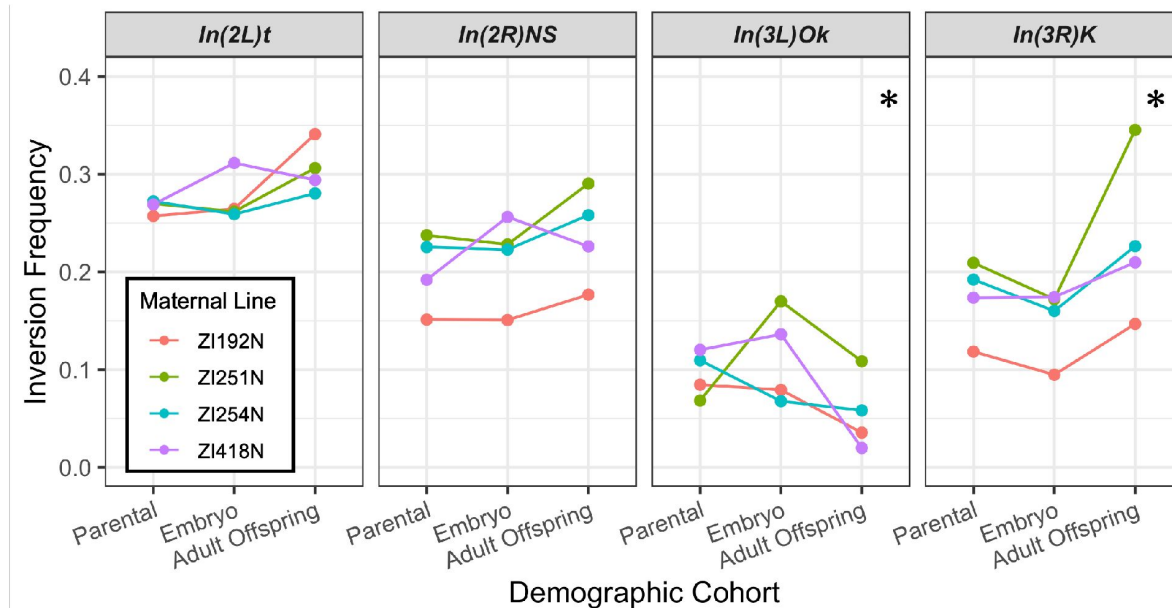


Figure 8

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

A Testing for tradeoffs between male reproduction and survival



B Inversions convey some sex-specific survival effects

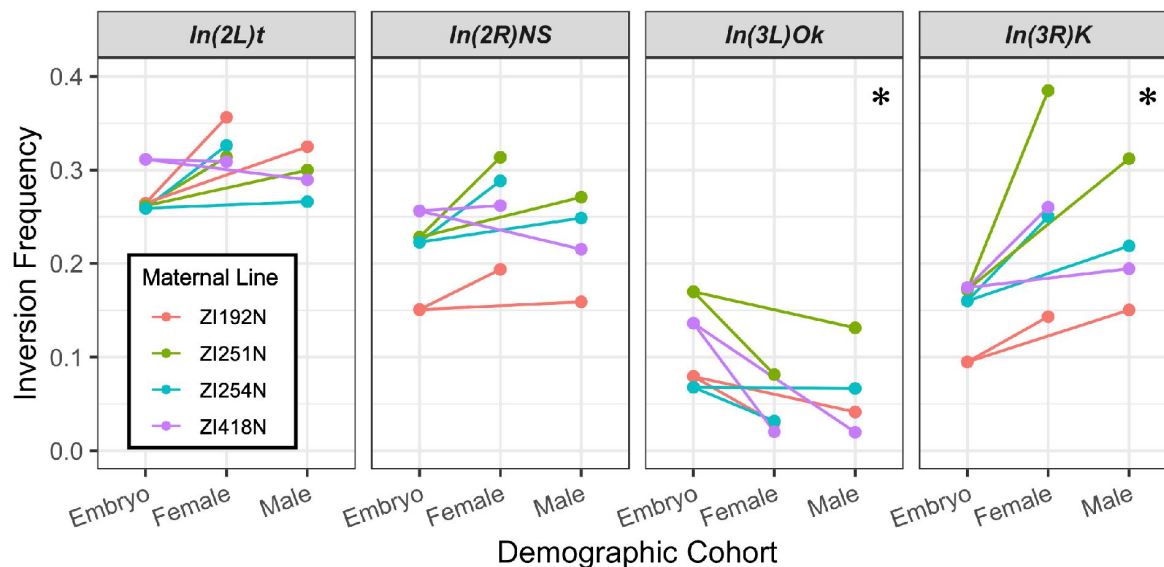


Figure 9

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

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 often context-dependent influences on male reproduction and on survival, with some evidence for antagonistic tradeoffs of the sort proposed in our model.

3 Discussion

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

3.1 Inversions in *D. melanogaster*

We have presented results from a single generation laboratory evolution experiment, in which we crossed outbred male *D. melanogaster* carrying inversions *In(2L)t*, *In(2R)NS*, *In(3L)Ok*, and *In(3R)K* to four separate inbred female lines and tracked the frequency of the inversions in the initial males, embryonic offspring, and aged adults. For most inversions, the strength and direction of the tradeoff varied notably between crosses to different inbred maternal lines, mostly due to maternally-dependent variation in male reproductive-success. However, greater consistency was

observed for *In(3R)K*, which was associated with lesser male reproductive success but greater survival. A significant tradeoff was also inferred for *In(3L)Ok*, which favored male reproduction (albeit variably) over survival. These results provide significant but measured evidence for the presence of sexually antagonistic autosomal inversions in this model species, while emphasizing the importance of genetic background.

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

This model could also help explain the observed geographic variability of inversion frequencies, as a change in environment may change the fitness value of either pleiotropic effect of the alleles. If a population experiences a novel harsh environment with low survivability, then the population density may decrease, and the number of encountered competing males may decrease as well, reducing the relative fitness advantage of enhanced male display. If there is a genetic tradeoff between mating display and viability, the equilibrium frequency of an antagonistic inversion that favors the genetic extreme of display should be lower. Additionally, in hostile environments, the survival cost of display-favoring variants could be increased, which could further shift their equilibrium frequencies downward or render them not worth carrying at any frequency. These predictions could contribute to the observed negative relationships between some inversion frequencies and latitude or altitude (Kapun et al. 2016 [↗](#); Pool et al. 2017 [↗](#)). As a specific example, it is notable that in a high altitude Ethiopian environment with year-round cool weather, which hosts some of this species' most pronounced phenotypic evolution (Bastide et al. 2014 [↗](#); Lack et al. 2016b), inversions are virtually absent - occurring at the lowest frequencies of any analyzed population (Sprengelmeyer et al. 2020 [↗](#)). Consistent with such reasoning, a disadvantage of some inversions in challenging thermal environments (both cold and hot) was supported by a previous experimental evolution analysis (Kapun et al. 2014 [↗](#)). However, our experimental results include evidence that some inversions, such as *In(3R)K*, may have the opposite tradeoff - that the inverted arrangement is associated with survival success instead of reproductive success. Notably, we have only tested survival and reproduction in a benign laboratory environment, and not under challenging environments such as cold temperatures. Other studies in *D. melanogaster*, including those on focused seasonal evolution, have found evidence for important tradeoffs between reproduction and robustness to environmental challenges (e.g. Behrman et al. 2015 [↗](#)). Hence, it would clearly be desirable to study the effects of inversions on a wider range of potential tradeoffs. For example, Said et al. (2018) [↗](#) suggested that inversions *In(2L)t* and *In(3R)K* might be maintained by immunity-related tradeoffs, in light of an over-representation of immune-related genes among differentially expressed transcripts between inverted and standard karyotypes.

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

As indicated in the Introduction, balancing selection on inversions could also be achieved by ‘associative overdominance’ (Zhao and Charlesworth 2016 [↗](#); Gilbert et al. 2020 [↗](#)), where two haplotypes are balanced by recessive cost to both homozygotes. Inversions may be particularly susceptible to carrying recessive deleterious 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.

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

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

Essentially, any population with antagonistic pleiotropy involving some form of negative frequency-dependent 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 chromosome system, suggesting that this particular pleiotropic trade-off may favor resolution by linkage to a female sex determination factor. Further, simulations in which only females experienced antagonistic survival cost usually generated populations with a chromosome essentially lethal in females, thus exclusively transmitted in males similar to a Y chromosome (**Figure 7** [↗](#)). These simulations sometimes had three states that appeared stable at 20N generations, including Y-like, W-like, and shared intermediate arrangements. This three-arrangement stable polymorphism seems unusual for a natural population, but might be interesting to explore in populations with polymorphic sex determination.

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

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

Summary and Future Prospects

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

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

4.2 Experimental Populations and Sequencing

To act as a high-inversion source population for the potential fathers in our experiment, we combined 15 males and 15 females from each of 10 inbred strains derived from wild flies collected in Zambia. These 10 strains were chosen to collectively carry the following common inversions: *In(2L)t*, *In(2R)NS*, *In(3R)K*, *In(3R)P*. We then collected at least 500 F2 males from this high-inversion population to use in each of our independent maternal line experiments, and these males were expected to carry a wide range of inversion genotypes. Each batch of high-inversion males was crossed to an equal number of virgin females from a single inbred Zambian strain (Figure 6). Four such experiments were performed, each using one inversion-free maternal strain. Since no inversions could be inherited through the mothers, inversion frequencies of successfully reproducing F2 males could be inferred from their pooled offspring. Unless otherwise noted, all flies were kept in a lab space of 23°C with around a degree of temperature fluctuation and without a controlled day/night cycle. All fly food used was prepared in batches of 4.5 L water, 500 mL cornmeal, 500 mL molasses, 200 mL yeast, 54 g agar, 20 mL propionic acid, and 45 mL tegosept 10% (in 95% ethanol). The four parental populations were each allowed to mate freely in clear plexiglass cages of 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 shut with a binder clip. The parents were given either fly food bottles or grape agar in 7.9 cm petri dishes as food or laying substrate on alternating days over two weeks, after which all surviving male parents were counted and frozen. The grape agar was prepared in batches of 500 mL from FlyStuff.com grape agar powder, and wet Red Star yeast was brushed over the agar plates before use to encourage laying. Embryos were collected from the agar, counted, and frozen for sequencing. Embryos laid in the bottles were allowed to hatch and develop into adults. These adult offspring were separated by sex and by the time after laying at which they eclosed. In our husbandry conditions, the earliest eclosion was 11 days after laying, but this was rare and a first eclosion at 12 days was more common among the bottles. Most flies took several days longer to develop, and we considered flies collected in the first 6 days of a 10 day collection window to be the early-eclosing pool, in order to split the pool sample sizes closer to evenly. The collected adult flies were then aged by sequentially transferring them between bottles weekly for approximately 2.5 months, and then the adult offspring were sexed, counted, and collected. A 10-week aging window was chosen based on the time taken for a control set of four bottles of flies from the paternal outbred population to reach 80% mortality. All adult and embryo samples were frozen at -80°C before DNA extraction. This sampling scheme was chosen to identify inversion frequencies in the set of potential parents, in embryonic offspring, and in older adults, and so to assess potentially selected changes in inversion frequency across male reproduction or in egg to adult viability plus longevity.

DNA for each replicate age group was extracted by a phenol-chloroform extraction protocol used previously in preparing DNA for the *Drosophila* Genome Nexus and *Drosophila* Population Genomics Project. The protocol is similar to protocol ‘BPC’ used in DPGP phase 1 (Langley et al. 2012), except with the addition of proteinase-K during homogenization of adult flies, and generally based on the common quick preparation protocol presented in *Drosophila Protocols* (Huang et al. 2009). Inversion genotyping was performed by amplicon sequencing, where amplicons associated with each inversion were designed to target at least one single nucleotide polymorphism in the inversion interior that is fixed for alternate alleles between the inversion karyotypes, while still sharing conserved primer regions across karyotypes to prevent primer bias. Genetic variation in these inversions was assessed using previously sequenced and inversion-called haploid genomes from the Zambian *D. melanogaster* population, available from the *Drosophila* Genome Nexus (Lack et al. 2016a). The amplicon region and primer selection was performed with a custom script. The script calls Primer3 version 2.4.0 for primer candidate generation (Koressaar and Remm 2007; Untergasser et al. 2012; Koressaar et al. 2018). Primers thus selected were ordered from IDT as oligos ligated to Illumina stubby adaptors [Table S1]. Illumina sequencing libraries were then prepared for each sample following the Illumina metagenomic amplicon sequencing protocol (Illumina 2013), with 50 ng/uL diluted DNA samples. and sequenced 2x150 on an Illumina NovaSeq to at least a depth of 20000 reads. Depth

was selected to ensure high likelihood of detecting a 0.025 change in inversion frequency from an initial frequency of 0.10, based on simulated resampling of the collected flies, DNA extraction, and sequencing.

4.3 Sequence Analysis and Statistics

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

To assess the significance of the difference in inversion frequency between two sampled life stages in the same experiment, we first calculated a chi-square test statistic from the two-by-two table of inverted and non-inverted allele counts at two life stages. Allele counts were taken by multiplying the inversion-specific SNP frequency in the sequencing reads by the allele sample size. We then generated an approximate distribution of the chi-square statistic when selection is absent (our null hypothesis), using a resampling approach (Figure 6 [DOI](#)). Comparing the observed statistic to our modeled neutral distribution of test-statistic values allowed us to identify significance thresholds.

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

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

In order to identify a frequency change reversal between life history stages that could reflect a fitness tradeoff, we tested for the joint significance of two opposing frequency changes using three cohorts. A tradeoff would require a frequency change in one direction between paternal and embryo samples, followed by a frequency change in the other direction between embryo and adult offspring samples. These changes both depend on the shared embryonic cohort in the test, and so are not independent. Therefore, we evaluated the two inversion frequency changes jointly from a set of resampled neutral data sets in which the three cohorts arose from the same resampling process. Here we considered how likely it was that a sample of the three cohorts from the associated estimated neutral model had chi-square values for both the comparison of the fathers to embryos and the embryos to combined adults that were equivalent or more extreme in the test direction than the observed data.

For example, if we were testing the significance of an observed paternal-embryo increase and then embryo-adult decrease in frequency, our p-value would be defined by the proportion of resampled replicates in which the frequency changes were both in these same directions, and their chi-square values were both more extreme than the empirical values.

To assess the significance of inversion frequency changes considered across all four maternal line crosses at once, 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/>.

Acknowledgements

Particular thanks to Shimeng Gao, who assisted with *Drosophila* husbandry and collection of adult flies during the within-generation experiment. We also thank members of the Pool lab for helpful comments on earlier versions of this manuscript.

This research was supported by National Science Foundation Graduate Research Fellowship (DGE-1747503 to CSM), and by the National Institutes of Health (grants R35 GM136306 to JEP, T32 GM007133, and T32 HG002760).

This research was performed using the compute resources and assistance of the UW-Madison Center for High Throughput Computing (CHTC) in the Department of Computer Sciences. The CHTC is supported by UW-Madison, the Advanced Computing Initiative, the Wisconsin Alumni Research Foundation, the Wisconsin Institutes for Discovery, and the National Science Foundation, and is an active member of the Open Science Grid, which is supported by the National Science Foundation and the U.S. Department of Energy's Office of Science.

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Reviewer #1 (Public Review):

The idea is that inversions capture genetic variants that have antagonistic effects on male sexual success (via some display traits) and survival of females (or both sexes) until reproduction. A series of simulations are presented and show that the scenario works at least under some conditions. While a polymorphism at a single locus with large antagonistic effects can be maintained for a certain range of parameters, a second such variant with somewhat smaller effects tends to be lost unless closely linked. It becomes much more likely for genomically distant variants that add to the antagonism to spread if they get trapped in an inversion; the model predicts this should drive the accumulation of sexually antagonistic variants on the inversion versus standard haplotype, leading to the evolution of haplotypes with very strong cumulative antagonistic pleiotropic effects. This idea has some analogies with one of the predominant hypotheses for the evolution of sex chromosomes, and the authors discuss these similarities. To provide empirical support for this idea, the authors study the dynamics of inversions in population cages over one generation, tracking their frequencies through amplicon sequencing, from the parental generation through embryos to aged adults of either sex. Out of four inversions included in the experiment, two show patterns consistent with antagonistic effects on male sexual success (competitive paternity) and the survival of offspring, especially females, until old age, which the authors interpret as consistent with their theory.

This is an interesting idea, and the authors should be praised for combining a model with experimental data. However, in addition to the potential for improvement of presentation (details below), the study has some substantial weaknesses that could be addressed with additional simulations and additional experiments.

(1) The authors claim that the negative frequency dependence that maintains polymorphism in their model results from a non-linear relationship between the display trait and sexual success. I am not convinced about that. It seems to me that the "best of n " female choice implemented in the model (l. 741ff and Figure 2) does not lead to negative frequency dependence. Let p be the frequency of the competitively inferior male genotype. Assuming no noise in the male display, a female will mate with an inferior male only if all males among the n males sampled by the female are of the inferior genotype, which will be the fraction p^n , the remaining $1-p^n$ matings will go to the superior males. Thus, per capita, the inferior males will achieve $(p^n)/p$ or $p^{(n-1)}$ matings while the per-capita matings per superior male will be $(1-p^n)/(1-p)$. Thus, the ratio of the mating success of the inferior to the superior males will be $(1-p) p^{(n-1)} / (1-p^n)$. For the range of p from 0 to 1, this is an increasing function of p . E.g., with $n = 2$, the sexual fitness of the inferior genotype relative to that of the superior phenotype is $p/(1+p)$. Thus, at least in the absence of noise in the mate choice, this generates positive rather than negative frequency dependence. Maybe I missed something, but the authors do not provide support for their claim about the negative frequency-dependence of sexual selection in their simulations. To do so they could (1) extract the relationship between the relative mating success of the two male types from the simulations and (2) demonstrate that polymorphism is not maintained if the relationship between male display trait and mating success is linear.

(2) The authors only explore versions of the model where the survival costs are paid by females or by both sexes. We do not know if polymorphism would be maintained or not if the survival cost only affected males, and thus if sexual antagonism is crucial.

(3) The authors assume no cost to aneuploidy, with no justification. Biologically, investment in aneuploid eggs would not be recoverable by *Drosophila* females and thus would potentially act against inversions when they are rare.

(4) The authors appear to define balanced polymorphism as a situation in which the average allele frequency from multiple simulation runs is intermediate between zero and one (e.g., Figure 3). However, a situation where 50% of simulation runs end up with the fixation of allele A and the rest with the fixation of allele B (average frequency of 0.5) is not a balanced polymorphism. The conditions for balanced polymorphism require that selection favors either variant when it is rare.

(5) Possibly the most striking result of the experiment is the fact that for 14 out of 16 combinations of inversion x maternal background, the changes in allele frequencies between embryo and adult appear greater in magnitude in females than in males irrespective of the direction of change, being the same in the remaining two combinations. The authors interpret this as consistent with sexually antagonistic pleiotropy in the case of *In(3L)Ok* and *In(3R)K*. The frequencies of adult inversion frequencies were, however, measured at the age of 2 months, at which point 80% of flies had died. For all we know, this may have been 90% of females and 70% of males that died at this point. If so, it might well be that the effects of inversion on longevity do not systematically differ between the ages and the difference in Figure 9B results from the fact that the sample includes 30% longest-lived males and 10% longest-lived females.

(6) Irrespective of the above problem, survival until the age of 2 months is arguably irrelevant from the viewpoint of fitness consequences and thus maintenance of inversion polymorphism in nature. It would seem that trade-offs in egg-to-adult survival (as assumed in the model), female fecundity, and possibly traits such as females resistance to male harm would be much more relevant to the maintenance of inversion polymorphisms.

(7) The experiment is rather minimalistic in size, with four cages in total; given that each cage contains a different female strain, it essentially means $N=1$. The lack of replication makes

statements like " In(2L)t and In(2R)NS each showed elevated survival with all maternal strains except ZI418N" (l. 493) unsubstantiated because the claimed special effect of ZI418N is based on a single cage subject to genetic drift and sampling error. The same applies to statements on inversion x female background interaction (e.g., l. 550), as this is inseparable from residual variation. It is fortunate that the most interesting effects appear largely consistent across the cages/female backgrounds. Still, I am wondering why more replicates had not been included.

- <https://doi.org/10.7554/eLife.93338.1.sa2>

Reviewer #2 (Public Review):

Summary:

In their manuscript, the authors address the question of whether the inversion polymorphism in *D. melanogaster* can be explained by sexually antagonistic selection. They designed a new simulation tool to perform computer simulations, which confirmed their hypothesis. They also show a tradeoff between male reproduction and survival. Furthermore, some inversions display sex-specific survival.

Strengths:

It is an interesting idea on how chromosomal inversions may be maintained

Weaknesses:

General points:

The manuscript lacks clarity of writing. It is impossible to fully grasp what the authors did in this study and how they reached their conclusions. Therefore, I will highlight some cases that I found problematic.

Although this is an interesting idea, it clearly cannot explain the apparent influence of seasonal and clinal variation on inversion frequencies.

Specific points:

The simulations are highly specific and make very strong assumptions, which are not well-justified.

- <https://doi.org/10.7554/eLife.93338.1.sa1>

Reviewer #3 (Public Review):

Summary:

In this study, McAllester and Pool develop a new simulation model to explain the maintenance of balanced inversion polymorphism, based on (sexually) antagonistic alleles and a trade-off between male reproduction and survival (in females or both sexes). In support of the plausibility of this model, the authors use laboratory experiments on four naturally occurring inversion polymorphisms in *Drosophila melanogaster*, finding evidence for the existence of the above-mentioned trade-off in two out of the four cases.

Strengths:

1. The study develops and analyzes a new (*Drosophila melanogaster*-inspired) model for the maintenance of balanced inversion polymorphism, combining elements of (sexually) antagonistically (pleiotropic) alleles, negative frequency-dependent selection, and synergistic epistasis. To this end, the authors developed and used a new simulator (although it was not 100% clear as to why SLiM could not have been used as SLiM has been used to study inversions).

2. The above-mentioned model assumes, as a specific example, a trade-off between male reproductive display and survival; in the second part of their study, the authors perform laboratory experiments on four common *D. melanogaster* inversions to study whether these polymorphisms may be subject to such a trade-off. The authors find that two of the four inversions show suggestive evidence that is consistent with a trade-off between male reproduction and survival. The new amplicon sequencing approach to track inversion frequencies used by the authors seems promising in terms of studying fitness effects/trade-offs associated with polymorphic inversions and how such effects play out dynamically.

Weaknesses:

1. Mechanisms of balancing selection maintaining balanced inversion polymorphism. In Section 1.1 a better and more accurate overview of the different selective mechanisms that might contribute to the maintenance of balanced inversion polymorphisms should be given (for a recent review see Berdan et al. 2023). For example, negative frequency-dependent selection (NFDS), spatially and temporally varying selection are not mentioned here and are brought up only later, which is not really ideal. While I agree that in most cases our understanding of balanced inversion polymorphism is very limited, there are many empirical examples of these and other mechanisms of balancing selection being at play (e.g., NFDS: Wright & Dobzhansky 1946; Nassar et al. 1973; Álvarez-Castro and Álvarez 2005; Jay et al. 2021; and many examples of evidence for other mechanisms as well). Thus, while the prevalence of inversion polymorphisms is indeed in many cases "enigmatic", the reader should not be given the impression that we do not have yet any empirical evidence for specific mechanisms in particular cases. Similarly, the authors mention the classical (essentially Dobzhansky's) scenario of epistatically interacting loci only in passing, even though this coadaptation scenario may be simpler than the local adaptation mechanism of Kirkpatrick & Barton (2016): in its simplest form, epistatic coadaptation does not require any migration load or locally adaptive alleles à la Kirkpatrick & Barton, but just the capture of 2 overdominant loci, with inversion protecting this fittest double heterozygote from recombination load (Charlesworth 1974; also see Charlesworth & Charlesworth 1974; Charlesworth & Flatt 2021; also see discussion in Charlesworth and Barton 2018). On the other hand, the plausibility of the mutational load/associative overdominance (AOD) mechanism (Sturtevant & Mather 1938; Nei et al. 1967; Ohta 1971) seems to be given too much weight: new work by Charlesworth (2023; <https://www.biorxiv.org/content/10.1101/2023.10.16.562579v1>) suggests that load likely contributes only very modestly to heterokaryotypic advantage of inversions at intermediate frequencies, and that is very unlikely to provide a sufficient selective (heterotic) advantage to new autosomal inversions in order to explain their establishment (also see Nei et al. 1967; Connallon and Olito 2021; Jay et al. 2022).

2. The general reduction principle and inversion polymorphism. In Section 1.2., the authors state that "there has not been a proposed mechanism whereby alleles at multiple linked loci would directly benefit from linkage and thereby maintain an associated inversion polymorphism under indirect selection." Perhaps I am misunderstanding something, but in my reading, this statement is factually incorrect. In fact, the simplest version of Dobzhansky's epistatic coadaptation model (see Charlesworth 1974; also see Charlesworth and Charlesworth 1973 and discussion in Charlesworth & Flatt 2021; Berdan et al. 2023) seems to be an example of exactly what the authors seem to have in mind here: two loci experiencing overdominance, with the double heterozygote possessing the highest fitness (i.e., 2 loci under epistatic selection, inducing some degree of LD between these loci), with subsequent capture by an inversion; in such a situation, a new inversion might capture a haplotype that is present in excess of random expectation (and which is thus fitter than average). The selective benefits of recombination suppression in the inversion heterokaryotype will then confer a heterozygote advantage to the inversion and prevent it from going to fixation (see Charlesworth 1974). This is probably the simplest (or one of the simplest) models of

multilocus balancing selection that can act on inversions. Incidentally, this model represents a prime example of the "reduction principle", which the authors mention on two occasions in their paper: generally, any multi-locus polymorphism held at equilibrium by any type of balancing selection involving fitness epistasis will cause selection for reduced recombination (e.g., Feldman & Liberman, 1986; Zhivotovsky et al., 1994); notably, the example of inversion polymorphism is explicitly discussed in Altenberg's and Feldman's (1987) paper on the reduction principle. It is also noteworthy in this context that the 2-locus epistatic model of Charlesworth (1974) assumes constant fitness values/selection coefficients but actually leads to what one could call "apparent" frequency-dependent selection with different equilibria.

3. Trade-offs and antagonistic pleiotropy involved in maintaining inversions. Throughout the manuscript, previous work implicating trade-offs and/or antagonistic pleiotropy (AP) in the maintenance of inversion polymorphisms should be more adequately acknowledged and discussed - from the text as it currently stands one gains the impression that barely anything is known about the connection between inversions and pleiotropy/AP/trade-offs (e.g., Betrán et al. 1998; Mérot et al. 2020, which is cited but not really in the context of AP/trade-offs; Pei et al. 2023, etc.). The paper by Pei and colleagues is particularly relevant in the context of the present study: the authors find that the inverted allele has beneficial effects on male siring success and female fecundity but negative effects on survival. Generally, numerous studies have found that inversion polymorphisms have "pleiotropic" (albeit not always antagonistically pleiotropic) effects upon multiple fitness components (e.g., Etges 1989; Betrán et al. 1998; Küpper et al. 2016; Durmaz et al. 2018; Mérot et al. 2020). More broadly, the general role of AP in maintaining (life-history) polymorphisms should be mentioned by referring to previous theories (e.g., Rose 1982, 1985; Curtsinger et al. 1994; Charlesworth & Hughes 2000 chapter in Lewontin Festschrift; Connallon & Chenoweth 2019 - this latter paper is particularly relevant in terms of AP effects in the context of sexual antagonism).

4. Sexually antagonistic selection and inversion polymorphism. The authors' model of sexual antagonism being involved in maintaining an inversion polymorphism is novel and interesting, but again I felt that the authors' ideas could be better connected to what has been done before. First, several papers have made connections between sexual antagonism and inversion polymorphisms: for example, an important study that deserves discussion in this context is the paper by Natri and colleagues (2019) where the authors study sexual antagonism as a source of balancing selection that maintains an inversion polymorphism in the ruff. Similarly, another relevant study in this context is Hearn et al. 2022 on *Littorina saxatilis* snails. Also see Giraldo-Deck et al. (2022). A very interesting paper that may be worth discussing is Connallon & Chenoweth (2019) about dominance reversals of antagonistically selected alleles (even though C&C do not discuss inversions): AP alleles (with dominance reversals) affecting two or more life-history traits provide one example of such antagonistically selected alleles (also see Rose 1982, 1985; Curtsinger et al. 1994) and sexually antagonistically selected alleles provide another. The two are of course not necessarily mutually exclusive, thus making a conceptual connection to what the authors model here.

5. The model. In general, the description of the model and of the simulation results was somewhat hard to follow and vague. There are several aspects that could be improved: (1) it would help the reader if the terminology and distinction of inverted vs. standard arrangements and of the three karyotypes would be used throughout, wherever appropriate. (2) The mention of haploid populations/situations and haploid loci (e.g., legend to Figure 1) is somewhat confusing: the mechanism modelled here, of course, requires suppressed recombination in the inversion/standard heterokaryotype; and thus, while it may make sense to speak of haplotypes, we're dealing with an inherently diploid situation. (3) The authors have a situation in mind where the 2 karyotypes (INV vs. STD) in the heterokaryotype carry distinct sets of loci in LD with each other, with one karyotype/haplotype carrying antagonistic variants favoring high male display success and with the other karyotype/haplotype carrying non-antagonistic alternative alleles at these loci and which favor survival. Thus, at each of

the linked loci, we have antagonistic alleles and non-antagonistic alleles - however, the authors don't mention or discuss the degree of dominance of these alleles. The degree of dominance of the alleles could be an important consideration, and I found it curious that this was not mentioned (or, for that matter, examined). (4) In many cases, the authors do not provide sufficient detail (in the main text and the main figures) about which parameter values they used for simulations; the same is true for the Materials & Methods section that describes the simulations. Conversely, when the text does mention specific values (e.g., 20N generations, 0.22-0.25M, etc.), little or no clear context or justification is being provided. (5) The authors sometimes refer to "inversion mutation(s)" - the meaning of this terminology is rather ambiguous.

6. Throughout the manuscript, especially in the description and the discussion of the model and simulations, a clearer conceptual distinction between initial "capture" and subsequent accumulation / "gain" of variants by an inversion should be made. This distinction is important in terms of understanding the initial establishment of an inversion polymorphism and its subsequent short- as well as long-term fate. For example, it is clear from the model/simulations that an inversion accumulates (sexually) antagonistic variants over time - but barely anything is said about the initial capture of such loci by a new inversion.

- <https://doi.org/10.7554/eLife.93338.1.sa0>