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The microevolutionary response to male-limited X-chromosome evolution in *Drosophila melanogaster* reflects macroevolutionary patterns

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Abstract

Due to its hemizygous inheritance and role in sex determination, the X-chromosome is expected to play an important role in the evolution of sexual dimorphism and to be enriched for sexually antagonistic genetic variation. By forcing the X-chromosome to only be expressed in males over >40 generations, we changed the selection pressures on the X to become similar to those experienced by the Y. This releases the X from any constraints arising from selection in females and should lead to specialization for male fitness, which could occur either via direct effects of X-linked loci or trans-regulation of autosomal loci by the X. We found evidence of masculinization via up-regulation of male-benefit sexually antagonistic genes and down-regulation of X-linked female-benefit genes. Potential artefacts of the experimental evolution protocol are discussed and cannot be wholly discounted, leading to several caveats. Interestingly, we could detect evidence of microevolutionary changes consistent with previously documented macroevolutionary patterns, such as changes in expression consistent with previously established patterns of sexual dimorphism, an increase in the expression of metabolic genes related to mito-nuclear conflict and evidence that dosage compensation effects can be rapidly altered. These results confirm the importance of the X in the evolution of sexual dimorphism and as a source for sexually antagonistic genetic variation and demonstrate that experimental evolution can be a fruitful method for testing theories of sex chromosome evolution.

KEYWORDS

experimental evolution, gene expression, microarray, sexual conflict

1 | INTRODUCTION

Sex chromosomes have long been of interest to researchers in evolution and genetics, due to their special mode of inheritance and association with sex determination (Abbott, Nordén, & Hansson, 2017). In XY systems, the X-chromosome is expected to be feminized (enriched in female-biased genes compared to the autosomes) and/or demasculinized (impoverished for male-biased genes

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compared to the autosomes) because it spends two-thirds of its time in females, compared to only half for autosomes. Although there are exceptions, these predictions generally seem to hold true for species with heteromorphic sex chromosomes (reviewed in Dean & Mank, 2014). The X is also expected to be enriched for sexually antagonistic genetic variation, that is alleles with opposite effects on fitness between males and females (also known as intralocus sexual conflict). However, expectations from theories of sexual antagonism are slightly different from the predictions mentioned above, in that they predict that the X should be enriched for loci with recessive male-benefit and/or dominant female-benefit sexually antagonistic alleles (Rice, 1984, Patten, 2018; but see Fry, 2009). This is because any sexually antagonistic male-benefit allele will be expressed in hemizygous males, but masked in heterozygous females when recessive. Although this prediction is more challenging to test, it has some support (Connallon & Knowles, 2006; Dean, Perry, Pizzari, Mank, & Wigby, 2012; Gibson, Chippindale, & Rice, 2002).

These two phenomena are not mutually exclusive, and although these predictions (enrichment both of female-biased genes and of male-benefit loci on the X) may superficially appear to be conflicting, this is only the case if we assume that female-biased genes only benefit females and male-biased genes only benefit males. This is not necessarily the case; for example a gene that suppresses a male-specific pathway should be up-regulated in females, but this does not imply that the pathway itself is female-benefit. Consistent with this, Immonen, Sayadi, Bayram, and Arnqvist (2017) found that female seed beetles up-regulated expression of male-biased genes and down-regulated expression of female-biased genes after mating. Another important factor is timescale. New male-biased or male-benefit genes probably arise at equal rates across the genome, but given that many new mutations are recessive, X-linked loci have an initial advantage in that they may immediately be expressed in males and can be positively selected (Charlesworth, Coyne, & Barton, 1987). However, due to the fact that the X spends more time in females than in males, it may be a less favourable environment than the autosomes for male-benefit or male-biased genes (but see Patten, 2018). This leads to selection for such genes to relocate to the autosomes over evolutionary time, causing demasculinization of the X. Indeed, previous results have shown that young testis-specific genes tend to be located on the X more often than expected, but that old genes are less likely to be located on the X than expected (Zhang, Vibranovski, Krinsky, & Long, 2010). Similarly, Long, Vibranovski, and Zhang (2012) reviewed traffic to and from the X and found that genes often move from the X to the autosomes in both Diptera and mammals.

Hemizygous inheritance has other implications as well. Because males only have one copy of the X and females have two, males are expected to have half the expression at X-linked loci as females do, all else being equal. In many species, this asymmetry in expression seems to be disadvantageous, since various forms of dosage compensation have evolved (Chandler, 2017). For example, female mammals inactivate one X-chromosome, after which both sexes hyperexpress the X to maintain equal expression with the autosomes (Graves, 2016). In *Drosophila*, males instead hyperexpress the X directly, through the action of dosage compensation complexes that bind to high-affinity sites (Conrad & Akhtar, 2012). The dosage compensation complex prevents chromatin formation, allowing more efficient hyperexpression of X-linked loci close to the high-affinity sites. This seemingly elegant system may, however, constitute a constraint for males, in that it hinders the evolution of sex-specific expression, particularly in loci close to high-affinity sites. This has been demonstrated in a cross-species comparison, which found that male-biased genes tended to be located in regions without dosage compensation, farther away from high-affinity sites (Bachtrog, Toda, & Lockton, 2010). Finally, theory suggests that mito-nuclear interactions may be particularly likely on the X (Ågren, Munasinghe, & Clark, 2018), although empirical support for this prediction varies across species (Dean, Zimmer, & Mank, 2014). Nevertheless, Rogell, Dean, Lemos, and Dowling (2014) found that mito-sensitive genes (i.e. nuclear genes that changed in expression when paired with different mitochondrial genotypes) are underrepresented on the *Drosophila* X, even if nuclear genes with a mitochondrial annotation (i.e. mito-nuclear genes) are not (Dean et al., 2014).

Experimental alteration of mating regimes has been successfully employed to test hypotheses related to sexual selection and sexual conflict, and their effect on gene expression (Hollis, Houle, Yan, Kawecki, & Keller, 2014; Immonen, Snook, & Ritchie, 2014; Innocenti, Flis, & Morrow, 2014; Perry et al., 2016). Some of these studies support the idea that elevated levels of sexual selection/conflict result in a shift towards the male optimum via an increase in expression of male-biased genes (e.g. Hollis et al., 2014), but others do not (e.g. Immonen et al., 2014; Veltsos, Fang, Cossins, Snook, & Ritchie, 2017). In contrast, studies of sex chromosome evolution are often observational or comparative in nature (e.g. Mank & Ellegren, 2009; Wright et al., 2015), due to the difficulty in constructing experimental tests of macroevolutionary patterns (although there are some exceptions; Dean et al., 2012). Manipulative experiments testing predictions about sex chromosome evolution are therefore particularly valuable, since they make it possible to disentangle causal effects from stochastic effects (Abbott et al., 2017; Kawecki et al., 2012). We carried out a male-limited X-chromosome evolution experiment in *Drosophila melanogaster* designed to integrate predictions from both sexual antagonism and sex chromosome evolution, where X-chromosomes were passed from father to son for >40 generations and never expressed in females. This experimental protocol changes the selection pressures on the X to become similar to those on the Y-chromosome and should result in specialization of evolved X's to enhance male fitness. It also decouples inheritance of the X and the mitochondria, which may have implications for mito-nuclear conflict. We therefore expected to see masculinization at the phenotypic level, as well as in the expression profile via up-regulation of male-benefit genes and down-regulation of female-benefit genes in the selected populations. Based on previous evidence of sexual antagonism in this species, we also expected to see antagonistic changes in fitness in males and females (Prasad, Bedhomme, Day, & Chippindale, 2007).

Overall, our results were consistent with these predictions, although there were some surprises. Evidence of masculinization and sexual antagonism was relatively weak on the phenotypic level but there were clear signatures of both on gene expression. We were also able to detect a change in locomotory activity consistent with previous characterization of this trait as sexually antagonistic (Long & Rice, 2007). Interestingly, Gene Ontology analysis suggested changes in some traits which are not previously characterized as sexually antagonistic in this species but likely to be important in sexual selection, such as vision and learning/memory. An exciting finding, given the short timescale of the experiment, was evidence of microevolutionary changes consistent with the patterns of macroevolutionary change discussed above, including up-regulation of male-biased genes and down-regulation of female-biased genes in the selected populations, change in the expression of metabolic genes related to mito-nuclear conflict and evidence that dosage compensation may constitute a constraint for male-benefit genes. These results suggest that we were successful in releasing males from constraints arising from selection in females and therefore experimentally manipulating the selection pressures on the X-chromosome.

2 | METHODS

2.1 | Experimental protocol

All populations were derived from the LH_M stock (Chippindale & Rice, 2001), which is a large, outbreeding population with an easily replicable maintenance regime (see Supplementary Information for more details). Fly lines were maintained so as to follow the ancestral culturing protocol as closely as possible. In order to control inheritance of X-chromosomes and limit expression of the X to males, we used compound X (i.e. 'double X' or DX) females. DX females have two marked X-chromosomes that are linked at the centromere (*C(1) DX, y, f*) and were backcrossed to the LH_M stock population for 6 generations before the start of the experiment, so that they carried a random LH_M Y-chromosome and LH_M autosomes which were >98% identical to the base stock. When wild-type males are mated with DX females, X-bearing sperm can fertilize Y-bearing eggs, resulting in father–son transmission of the X (autosomal inheritance is unaffected; Figure 1). Six populations were started simultaneously; three replicate male-limited X-chromosome (MLX) populations and three control populations. All populations started by mating 480 males from the LH_M stock population to an equal number of females. In the control populations, these females were wild-type LH_M females, and in the MLX populations, they were the backcrossed DX females. The total initial population size was therefore 960 individuals, but this was later reduced to 640 individuals (320 of each sex) for logistical reasons (Abbott, Innocenti, Chippindale, & Morrow, 2013). The X-chromosome population size was inevitably lower in the MLX populations compared to the control populations under this protocol, since females in the MLX populations do not carry wild-type X-chromosomes. However, adjusting for this difference would have resulted in large differences in the amount of autosomal variation available between the treatments, so we instead chose initial population sizes deemed large enough to provide a reasonable level of X-linked standing genetic variation. Because the X is in a hemizygous state in males, we also simultaneously started a 'recombination box' treatment for each replicate MLX population (Abbott et al., 2013; Prasad et al., 2007). The recombination box serves to introduce sufficient recombination to reduce the effects of linkage, such as background selection and hitchhiking. See the Supplementary Information for more information.

2.2 | Phenotypic assays

After 40 generations of experimental evolution, we measured male fitness using a standard eye-marker protocol (Abbott, Bedhomme, & Chippindale, 2010; Abbott et al., 2013), in which male fitness is measured as proportion adult offspring produced when in competition with other males. See the Supplementary Information for more details. Target males were taken directly from the MLX and control populations, as were control females. However, wild-type females are not normally produced within the MLX populations, so they were obtained by first crossing MLX males with females from the control population of the same number. This results in females with one MLX X-chromosome and one control X-chromosome, and

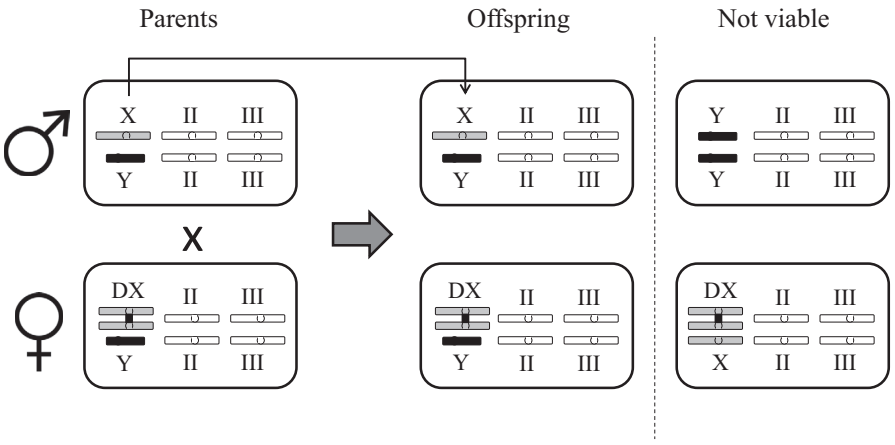

FIGURE 1 MLX evolution protocol. Males are mated to females with a double X-chromosome (DX), which forces father–son transmission of the X-chromosome and produces wild-type males with a paternally inherited X-chromosome and a maternally inherited Y-chromosome. New DX females with a paternally inherited Y-chromosome are also produced. Triple-X and double-Y individuals are not viable. From Abbott et al., 2013.

one set of autosomes from each treatment. Although the observed effects of the MLX selection on females might have been larger if using females that have two MLX X-chromosomes, this also admits the possibility of (likely deleterious) inbreeding effects on the X. We therefore elected to use females that were heterozygous with respect to X-chromosome origin, in order to avoid confounding inbreeding effects with sexual antagonism. Similarly, the mixed autosomal origin in females means that our results are likely to be conservative if any correlated changes have occurred on the autosomes as a result of MLX evolution. Another advantage of this approach is that differences between females will not be confounded with maternal effects, since both control and MLX assay females have control mothers.

We were also interested to see whether MLX evolution had effects on sex-specific survival from egg to adult and therefore also collected data on offspring sex ratios when measuring fitness. For female data, test tubes were kept after eggs were counted and total number of adult offspring was later recorded in order to provide some data on total survival rates. Finally, based on results from the gene expression analysis, indicating that locomotory activity might be of interest (see below), we also collected data on that trait across sexes and selection treatments. We used a protocol from a previous study of locomotory activity in the LH_M population (Long & Rice, 2007). In the set-up, flies were kept in mixed-sex groups in which the target sex is wild type and the opposite sex is brown-eyed. Density was kept at the same level as in the adult competition vials, 16 individuals of each sex. In order to avoid observer bias (e.g. that the eye is drawn to motion), vial volume was divided up into 6 sections and the section to be observed was determined using a random number generator. The first target fly detected in the section was then observed for 8 s and scored for locomotory behaviour (binary 0/1 score if walking behaviour was observed or not). If no target fly was found in the selected section, then the observation was immediately terminated and a new random number was generated. Observations were repeated until all vials have been observed 10 times, and an average score per vial (i.e. proportion active flies) was used for further analysis.

In the statistical analysis of the phenotypic data, we preferred to carry out the analysis on population means since the population is the appropriate level of replication for this experiment. For these analyses, total sample size was always 12 (two sexes*two treatments*three replicate populations). However, results from an alternative approach using raw data with population as a random factor nested within treatment were qualitatively similar in most cases and are reported in the Supplementary Information. Means were calculated from data for 10 vials per sex, selection treatment and replicate population for fitness, sex ratio and survival. Since vials means are already based on ten repeated observations in the locomotory activity assay, only five vials per sex, selection treatment and replicate population were used. Fitness data were mean-zero unit variance standardized to allow comparison between the sexes and analysed with a two-way ANOVA with sex and selection treatment as fixed factors. Because sex ratio and locomotory activity data are in the form of proportions, these were analysed using GLMs with a quasibinomial distribution and sex and selection treatment as fixed factors. Similarly, survival was analysed with a GLM with a quasibinomial distribution, but because survival was only measured in offspring from the female part of the fitness assay, selection treatment was the only factor in this analysis. Post hoc analysis of interaction effects was carried out using the 'lsmeans' package (Lenth, 2016). All analyses were carried out in R (R Team, 2014).

2.3 | Gene expression analysis

Gene expression data were collected after 50 generations of experimental evolution using microarrays using males and females produced in the same way as for the phenotypic assays. RNA was extracted with TRIzol (Invitrogen) and purified using an RNeasy Mini Kit (Qiagen). On day 12 from egg, two replicates of 8 individuals were taken for each combination of sex, selection treatment and replicate population. These flies were nonvirgin and kept under standard conditions for the experimental stocks until they were flash-frozen in liquid nitrogen. RNA quantity and quality was assessed using an Agilent Bioanalyzer (Agilent Technologies) prior to sample preparation and hybridization at the Uppsala Array Platform (using GeneChip Drosophila Genome 2.0 Affymetrix microarrays following the manufacturer's instructions). Note that the male samples in this analysis partially overlap with data published elsewhere (Abbott et al., 2013), but the data sets were normalized and analysed separately, and results suggest that these two sets of analyses can be considered independent in terms of their output (see Supplementary Information).

Analysis was carried out in BioConductor (http://www.bioconductor.org), and data were preprocessed using Robust Multichip Average (RMA) in the 'affy' package (Gautier, Cope, Bolstad, & Irizarry, 2004). Significant differences in gene expression levels were tested using a model that included selection treatment, sex and their interaction, as well as replicate population to control for nonindependence of replicate samples, with a false discovery rate (FDR) correction at 0.05. Replicate population was considered a random factor nested within treatment. In line with other studies of expression changes after experimental evolution (Immonen et al., 2014; Veltsos et al., 2017), we did not employ a fold change cut-off since most changes were expected to be relatively small.

Once genes that had changed significantly in expression between selection treatments had been identified, they were then divided into down-regulated and up-regulated sets (in MLX relative to Control populations) and these sets were further investigated separately. In cases where gene lists were very short (e.g. genes that showed a significant interaction effect), properties were examined manually using information from online databases from the National Center for Biotechnology Information (NCBI; https://www.ncbi.nlm.nih.gov/). Up-regulated and down-regulated genes were tested for Gene Ontology (GO) terms, chromosomal distribution, tissue

specificity and association with sex-specific fitness and sexual antagonism, as measured in a previous study of the LH_M population (Innocenti & Morrow, 2010). Overrepresentation of GO categories was analysed using hypergeometric testing ('hyperGTest' in R), which tests whether particular GO terms are associated with a gene set more often than expected by chance. Chromosomal distribution was tested using a chi-squared test ('chisq.test' in R). Chromosomal location tests were run both with and without including chromosome 4, since this chromosome is nonrecombining and may skew results of the chi-squared analysis. Tissue specificity was measured in the same manner as in previous expression analyses of the LH_M population (Abbott et al., 2013; Innocenti & Morrow, 2010). Tissue specificity was previously defined as >2-fold difference in expression between tissues (Innocenti & Morrow, 2010), and overrepresentation of tissue-specific genes among the genes that responded to the selection treatment was tested using Fisher exact tests with Bonferroni correction for multiple testing. Association with sex-specific fitness and sexual antagonism was analysed using two-tailed mean-rank gene set enrichment (MR-GSE) tests (Abbott et al., 2013; Innocenti & Morrow, 2010).

2.4 | Relationship to extant sexual dimorphism

We tested whether change as a result of the selection treatment was in the same direction as existing expression differences between the sexes (i.e. whether MLX selection had a masculinizing effect, such that male-biased genes became even more up-regulated and female-biased genes became even more down-regulated relative to control populations). For this, we ran a model of the effect of sex on expression in the control samples only and then compared direction of change in the selected populations with direction of sexual dimorphism in the control population using a chi-squared test. Although these data could potentially also be analysed with a linear model, distributions of the datapoints are bimodal due to exclusion of nonsignificant expression differences (see Figure 2c), making a chi-squared test more appropriate. We checked whether associations with sex bias were robust to source population differences by testing whether sex-biased genes (as reported in the Sebida database, Gnad & Parsch, 2006) and sexually discordant loci (Stocks, Dean, Rogell, & Friberg, 2015) were overrepresented among genes that responded to the selection

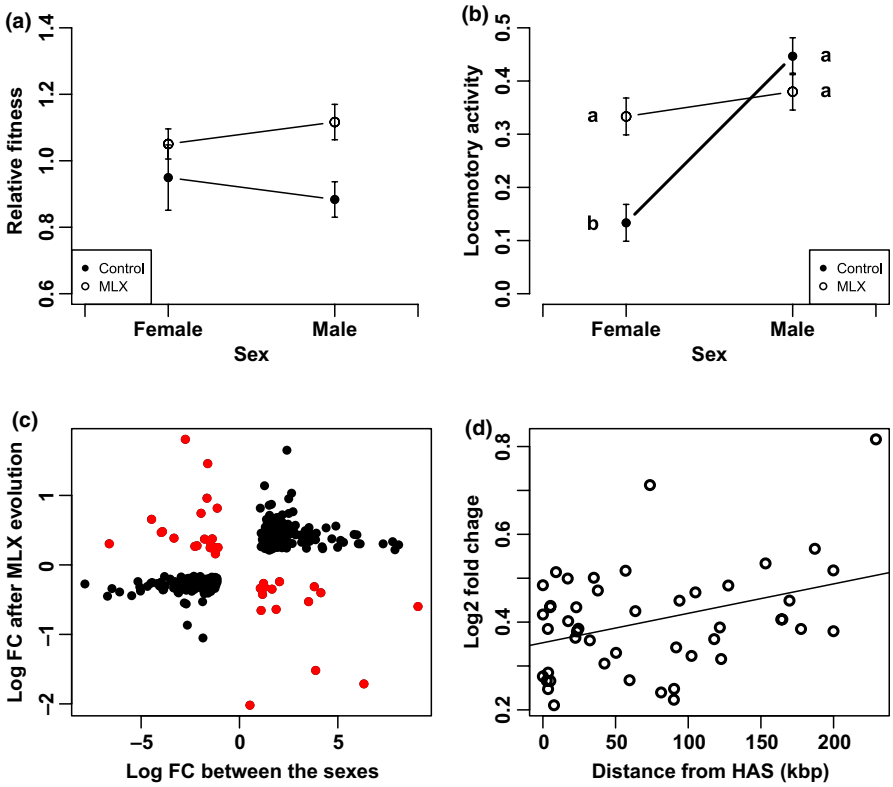

FIGURE 2 Overview of main results. (a) Fitness in relation to selection treatment and sex. MLX males have higher fitness than control males, but the treatments overlap in females. (b) Locomotory activity in relation to experimental evolution treatment and sex. Homogenous subsets are indicated with letters. MLX females have activity levels that are not significantly different from male activity levels. (c) Comparison of direction of sexual dimorphism in expression in control populations, with change in selected populations. Positive values on the *x*-axis indicate male-biased expression, and positive values on the *y*-axis indicate up-regulation after MLX evolution. Changes as a result of MLX evolution are much more often in the same direction as extant sexual dimorphism (black) compared to being in opposite directions (red). (d) Relationship between change in expression after MLX evolution and distance from the closest high-affinity site (HAS). High-affinity sites are associated with dosage compensation, and genes that were located farther away from high-affinity sites appear to be less constrained in their response to the selection treatment

treatment using chi-squared tests (Collet et al., 2016). We also tested for enrichment of genes more recently reported as sexually antagonistic based on allelic variation rather than expression differences (Ruzicka et al., 2019) in this data set, as well as for enrichment of genes that have previously been shown to change expression in response to manipulations of the intensity of sexual conflict (Immonen et al., 2014; Innocenti et al., 2014). Enrichment was again tested using chi-squared tests. Finally, we also tested the hypothesis that differences between selection treatments were smaller in females compared to males, using paired *t* tests of the absolute value of the difference for each transcript.

Male-biased genes are more likely to be located outside dosage compensation regions (Bachtrog et al., 2010), so we hypothesized that there might be a relationship between distance to high-affinity sites (associated with dosage compensation) and change in expression level as a result of the selection treatment using linear models. Location of high-affinity sites was obtained from Straub, Grimaud, Gilfillan, Mitterweger, and Becker (2008). Visual inspection of the relationship between change in expression and distance to the nearest high-affinity site revealed a potential outlier in the up-regulated data set, and this was confirmed using a standard diagnostic (Cook's distance > 1). Results excluding this outlier are therefore reported here, but the relationship is still highly significant if it is included.

2.5 | Mito-nuclear conflict and interaction analyses

We also checked whether any of the transcripts identified as having changed as a result of male-limited selection showed enrichment of genes previously identified as potentially subjected to mito-nuclear conflict using chi-squared tests (Rogell et al., 2014). Finally, two interaction network analyses were carried out: firstly to investigate degree of overlap with a previous data set and secondly to determine whether most significant genes fall into several large or many small clusters. Details of these interaction analyses are included in the Supplementary Information.

3 | RESULTS

3.1 | Phenotypic assays

Fitness was standardized by sex before analysis, so there was no effect of sex on fitness (as expected) and no significant interaction between sex and selection treatment. However, there was a main effect of selection treatment on fitness (p = .039, Table S1), such that MLX populations had higher fitness than control populations. Although the interaction term was not significant, the difference between the treatments was larger for males than for females (Figure 2a). This difference is confirmed if males and females are analysed separately; there is no difference between treatments in females ($F_{1,4}$ = 0.7393, p = .4384), but there is a significant difference in males ($F_{1,4}$ = 9.5474, p = .0366).

There was a significant effect of the interaction between sex and selection treatment on offspring sex ratio (p = .0009), as well as significant main effects (Table S2). Sex ratio was female-biased overall, but the MLX populations produce a higher proportion male offspring than control populations. This difference is especially noticeable for offspring of females with an MLX X-chromosome (Figure S2). A slightly female-biased sex ratio is not unusual for the LH_M population (Long & Pischedda, 2005), but the effect was likely compounded by overcrowding that occurred as a result of higher-than-anticipated female fecundity for this assay. In addition, the lower values overall for females with an MLX X-chromosome are a result of the fact that, for logistical reasons, these vials were counted two days later than the male vials. Some additional male mortality appears to have occurred during this period, which made us unable to distinguish the eye colour in the offspring and unambiguously assign male offspring to targets or competitors. The magnitude of the advantage of an MLX X-chromosome to male survival is therefore probably an overestimate relative to normal culturing conditions.

There was a significant effect of selection treatment on offspring survival ($p = 5.41*10^{-05}$), where offspring of MLX females had higher survival than offspring of control females (Table S1, Figure S3). Based on the results from the sex ratio analysis, it seems likely that this difference is a result of increased survival in sons who inherit an MLX X-chromosome, assuming that the sex ratio at fertilization is the same across selection treatments.

There was a significant effect of the interaction between sex and selection treatment on adult locomotory activity ($p = 7.23*10^{-05}$), as well as a significant main effect of sex (Table S2). Males had higher locomotory activity than females overall, but this activity was increased in MLX females, such that they were as active as males (Figure 2b). It is not clear why no difference between treatments was observed in males, but could be because males engaged to larger extent in other activities that were not measured, such as courtship or preening.

Results from the alternative approach using raw data with population as a random factor nested within treatment were qualitatively similar in all cases, except for fitness (Table S3).

3.2 | Changes in gene expression

6,286 transcripts were variable in expression across samples and were retained for analysis. Of these, 6,276 showed a significant effect of sex and 518 a significant effect of selection treatment. There was only one transcript showing a significant selection effect, that did not also show a significant sex effect (CG14957, annotated as being related to chitin production https://www.ncbi.nlm.nih.gov/gene/38386). This suggests that most of the variation across samples is related to sexual dimorphism. Only 4 transcripts showed a significant interaction effect between sex and selection treatment: CG10514 gene product from transcript CG10514-RA (CG10514), glutaminyl cyclase (QC), PCI domain-containing protein 2 (PCID2) and sallimus (sls). Because there were so few interaction effects, only

transcripts showing an effect of the selection treatment are considered further. We report results for up-regulated and down-regulated transcripts separately (i.e. increased or decreased in MLX lines compared to control), since they proved to be qualitatively different.

3.3 | Up- versus down-regulated transcripts

Of the 518 transcripts that responded significantly to the selection treatment, 342 were up-regulated and 176 were down-regulated in MLX relative to control populations. Log_2 fold changes ranged from ~0.15 to approximately 2.

Both up-regulated and down-regulated transcripts were distributed nonrandomly across chromosomes. Up-regulated genes were slightly underrepresented on the X but considerably enriched on chromosome 4 (χ^2 = 94.9102, *df* = 3, *p* < 2.2e-16, Table 1). This significant result is apparently driven by the enrichment on chromosome 4, since the difference is no longer significant when chromosome 4 is excluded from the analysis (χ^2 = 0.7212, *df* = 2, *p* = .6973). Down-regulated genes were underrepresented on chromosome 2 but enriched on the X-chromosome, consistent with the expectation that female-benefit genes might be overrepresented on the X (Table 1). This pattern showed a trend towards significance when all data were included (χ^2 = 6.4141, *df* = 3, *p* = .09311) and became marginally significant when chromosome 4 was excluded (χ^2 = 6.408, *df* = 2, *p* = .0406).

Up-regulated genes had tissue-specific expression (as previously characterized in Chintapalli, Wang, & Dow, 2007) more often than expected in the testes, ovaries, virgin and mated spermathecae, hindgut, fat body, heart and salivary glands (Table S4). Down-regulated genes showed tissue-specific expression in the crop only (Table S4).

GO terms for up-regulated and down-regulated genes are reported in Tables S5 and S6, respectively. Many of these were rather general and uninformative (e.g. 'System process', 'Signaling', 'Secretion'). Some interesting exceptions for up-regulated genes are terms associated with locomotory behaviour, metabolism, larval behaviour, phototaxis, learning and memory, and various terms related to vision (response to light stimulus, photoreceptor differentiation, detection of visible light). For down-regulated genes, there were a number of terms associated with DNA replication and damage repair, cell cycle regulation and oogenesis.

We tested whether genes that responded to the selection treatment were nonrandomly associated with fitness, as measured in a previous study (Innocenti & Morrow, 2010). We found that up-regulated genes were significantly associated with increased male fitness, decreased female fitness and sexual antagonism (Table 2). Down-regulated genes were significantly associated with increased female fitness and decreased male fitness, but there was no overrepresentation of genes characterized as sexually antagonistic (i.e. simultaneously increasing female fitness and decreasing male fitness or vice versa; Table 2).

3.4 | Relationship to extant sexual dimorphism

We found that changes in expression were overwhelmingly consistent with the direction of extant sexual dimorphism (483/518 in the same direction, versus 35/518 in the opposite direction, χ^2 = 387.5, *df* = 1, *p* = $2.2*10^{-16}$; Figure 2c). Similarly, sex-biased genes (both male-biased and female-biased as classified in SEBIDA) were overrepresented among the genes changed as a result of the selection treatment. Up-regulated genes were male-biased more often than expected (χ^2 = 237.8, *df* = 2, *p* = $2.2*10^{-16}$), and down-regulated genes were female-biased more often than expected (χ^2 = 193.1, *df* = 2, *p* = $2.2*10^{-16}$; Table 3). There was no evidence of enrichment of genes recently characterized as sexually antagonistic (all *p* > .07) by Ruzicka et al. (2019). Genes that have been previously shown to respond to altered intensity of sexual conflict in this species (Innocenti et al., 2014) were not overrepresented in this data set and were if anything underrepresented among down-regulated genes (χ^2 = 4.8698, *df* = 1, *p* = .02733). Genes that responded to altered intensity of sexual conflict in *Drosophila pseudoobscura*

TABLE 1 Chromosomal location of MLX transcripts

	Up-regulated transcripts		Down-regulated transcripts	
Chromosome	Observed	Expected	Observed	Expected
X	47	54.7	**40**	**28.2**
2	130	133.5	59	68.7
3	148	150	75	77.2
4	**16**	**2.1**	1	1.1

Note: Up-regulated transcripts were located on chromosome 4 significantly more often than expected (highlighted). Because chromosome 4 does not recombine, this is likely due to genetic hitchhiking. Down-regulated transcripts were located on the X-chromosome more often than expected (highlighted). This is consistent with the expectation that dominant female-benefit loci should often be located on the X.

TABLE 2 Relationship with fitness

Genes associated with	Up-regulated transcripts	Down-regulated transcripts
Male fitness	**Positive ($p = 1.9299*10^{-22}$)**	**Negative ($p = .0099$)**
Female fitness	**Negative ($p = 8.0925*10^{-07}$)**	**Positive ($p = 6.8031*10^{-07}$)**
Sexual antagonism	**Positive ($p = 2.2462*10^{-17}$)**	No association ($p = .3947$)

Note: Significant associations are shown in bold.

MLX transcripts show a pattern of association with fitness that is remarkably consistent with theory. Up-regulated transcripts are good for male fitness, bad for female fitness and significantly sexually antagonistic. Down-regulated transcripts are good for female fitness and bad for male fitness. Fitness data obtained from Innocenti and Morrow (2010).

(Immonen et al., 2014) were somewhat overrepresented in this data set (100 genes found where 74 were expected; $\chi^2 = 10.199$, $df = 1$, $p = .001405$). The effect of the selection treatment was larger in males compared to females, both for all genes in the data set and for the selected genes only ($t_{6285} = -31.154$ and $p < 2.2^{*}10^{-16}$ for all genes and $t_{517} = -24.623$ and $p < 2.2^{*}10^{-16}$ for selected genes). The effect was larger in the selected genes (mean reduction in fold change difference = 0.0764) compared to the full data set (mean reduction in fold change difference = 0.3252), consistent with the fact that significantly up-regulated genes were often male-biased.

Interestingly, we found a significant effect of distance to high-affinity sites (HAS) on expression of up-regulated genes. Increased distance from HAS regions resulted in greater up-regulation as a result of the selection treatment ($F_{1,45} = 7.2502$, $p = .0099$; Figure 2d). There was no overlap with genes previously identified as highly sexually concordant or discordant in their effect (Stocks et al., 2015).

3.5 | Mito-nuclear conflict

We examined three classes of genes potentially subjected to mito-nuclear conflict, after Rogell et al. (2014): mito-annotated genes (Gene Ontology ID 0005739), mito-sensitive genes (Innocenti, Morrow, & Dowling, 2011) and mito-proteome genes (Lotz et al., 2014). Although there was no signature of overrepresentation of these classes among the genes that responded to the selection treatment as a whole (for mito-sensitive genes: $\chi^2 = 2.179$, $df = 1$, $p = .14$; for mito-proteome genes: $\chi^2 = 0.076$, $df = 1$, $p = .78$), and mito-annotated genes were in fact slightly underrepresented ($\chi^2 = 4.108$, $df = 1$, $p = .043$), the pattern of regulation was revealed to be skewed. Both mito-annotated genes and mito-sensitive genes were up-regulated as a result of the selection protocol more often than expected by chance (mito-annotated: $\chi^2 = 7.629$, $df = 1$, $p = .0057$; mito-sensitive: $\chi^2 = 20.02$, $df = 1$, $p = 7.65^{*}10^{-6}$; Table 4). In addition, an analysis of chromosome location that was carried out for up-regulated mito-annotated genes (the only class of gene that had sufficient sample size for such an analysis) revealed that there was significant overrepresentation of genes located on chromosome 4 (6/44; $\chi^2 = 58.95$, $df = 3$, $p = 9.88^{*}10^{-13}$).

TABLE 3 Direction of change in expression after MLX evolution in relation to sex bias in gene expression

	Up-regulated transcripts		Down-regulated transcripts	
Sex bias	Observed	Expected	Observed	Expected
Male	**176**	**69.5**	13	47.2
Unbiased	49	89.1	4	55.3
Female	15	81.4	**146**	**60.5**

Note: Significant associations are shown in bold.

Male-biased genes are significantly overrepresented among up-regulated transcripts, and female-biased genes are significantly overrepresented among down-regulated transcripts.

4 | DISCUSSION

Here, we show that male-limited X-chromosome evolution affected phenotypic traits and gene expression in a way that was mostly, but not entirely, consistent with our initial predictions. We expected to see an antagonistic change in sex-specific fitness, but this prediction was not borne out because although male fitness increased, we did not find any concomitant decrease in female fitness. However, we did find evidence of an overall masculinization of locomotory activity, which is previously characterized as sexually antagonistic trait (Long & Rice, 2007). We also found considerable evidence of a masculinization of the expression profile in the selected populations and evidence that this response is in part a result of altered dosage compensation effects and release from mito-nuclear conflict.

4.1 | Caveats

There are several caveats to these results that should be kept in mind, at least some of which serve to make our analysis more conservative. Our experimental protocol made it impossible to keep the effective population sizes of the X and autosomes equal between the control and selected treatments. We elected to reduce the effective population size of the X in the MLX treatment in order to avoid confounding differences in autosomal standing genetic variation with the selection treatment. This reduction in X-chromosome population size should serve to limit the response to the selection treatment rather than enhance it. Similarly, due to logistical constraints at the time of expression data collection, we elected to use microarrays instead of RNAseq. RNAseq is superior for detecting low abundance transcripts, analysis of different

TABLE 4 Direction of change in expression after MLX evolution for three classes of mito-nuclear genes

	Mito-sensitive		Mito-annotated		Mito-proteome	
Regulation	Observed	Expected	Observed	Expected	Observed	Expected
Up-regulated	**44**	**29.3**	**6**	**2.6**	11	13.7
Down-regulated	**6**	**21.7**	**0**	**3.4**	20	17.3

Note: Significant associations are shown in bold.

Mito-sensitive and mito-annotated genes were up-regulated significantly more often than expected. There was no deviation from the null expectation for mito-proteome genes.

isoforms and generally produces lower amounts of technical variation compared to microarrays (Daines et al., 2011; Marioni, Mason, Mane, Stephens, & Gilad, 2008). This implies that our results are more likely to suffer from low resolution rather than extensive false positives (unfortunately, the lines are no longer available for complementary analysis using RNAseq for confirmation). Because changes in expression were investigated using whole-fly extractions rather than organs, we are also unable to distinguish between changes resulting from true up-regulation versus changes in relative organ size (allometric effects). This limits our ability to determine exactly which mechanism(s) caused the changes in expression levels, but is sufficient for achieving our main aim of detecting evidence of changes associated with traits previously characterized as sexually antagonistic.

A possible confound comes from the DX females used in the experimental evolution protocol, if the autosomes in the MLX treatment adapted to the presence of the DX and Y in females. Although we cannot entirely discount this possibility, any autosomal adaptations to, for example Y-induced male-specific chromatin remodelling patterns in females (Lemos, Branco, Jiang, Hartl, & Meiklejohn, 2014), or to increase female fitness in the presence of the DX, seem more likely to decrease the response to the MLX treatment than to alter the qualitative nature of the response. In addition, MLX females from the female fitness assay did not show evidence of reduced fitness when the DX was removed, which suggests no major effect of autosomal adaptation to the presence of the DX. Ideally, additional experiments could have been carried out to disentangle the effects of the X, evolved autosomes and Y-chromosome, but unfortunately this was not possible within the constraints of the project. However, because our results were generally consistent with predictions from theory and because we have no a priori reason to expect that coevolutionary effects should produce such results, on balance it seems likely that most of the response seen here was in fact due to altered selection pressures on the X-chromosome.

4.2 | Phenotypic data shows weak evidence of sexual antagonism

Several previous experiments have found that sex-limited selection leads to an increase in the fitness of the selected sex and a decrease in the fitness of the unselected sex in this species (Morrow, Stewart, & Rice, 2008; Prasad et al., 2007; Rice, 1992). Female-limited selection generally seems to have a smaller effect in the selected sex (~10% increase in Rice, 1992 and Morrow et al., 2008) than male-limited selection (~15% increase in Prasad et al., 2007 and Morrow et al., 2008). Interestingly, we found a larger increase in fitness in males (~25%, 95% confidence interval 2.36% to 44.2%) than in any of these previous studies. This is particularly surprising given that a smaller portion of the genome (i.e. the X) had the opportunity to respond to selection in this study compared to the whole-genome approaches of Prasad et al. (2007) and Morrow et al. (2008). This could indicate a large contribution of X-linked loci to male fitness, but may also be due to difference in number of generations (25–29 versus >40). The usual explanation for an associated decrease in fitness in the unselected sex is sexual antagonism, although mutation accumulation at sex-limited loci is also a possibility in long-term experiments. We were therefore surprised that we did not recover any signal of sexual antagonism in fitness (Figure 2a).

One possibility is that antagonism had been resolved in this population at the time of data collection (Collet et al., 2016). However, several lines of evidence suggest that antagonism may have existed but that we were unable to detect it here: other traits showed signatures of release from constraint imposed by selection in the other sex (e.g. locomotory activity and egg to adult survival, Figure 2b, Figures S2 and S3); there were signatures of phenotypic masculinization (Figure 2c); and changes in gene expression occurred in genes that were previously identified as sexually antagonistic (Table 3). There are then two plausible explanations for the somewhat puzzling lack of a decrease in female fitness. The first is that our experimental protocol for measuring female fitness did not capture all the relevant fitness variation. This is certainly possible, although fitness assays were carried out in such a way as to reflect fitness under the experimental culturing protocol and are similar to those used in previous studies of sexual antagonism in the population (Gibson et al., 2002; Prasad et al., 2007) and should therefore be relevant. However since any effects of the selection treatment on females were indirect, it is possible that there may have been mildly deleterious effects on female fitness that we did not have the power to detect, but which could have been better captured by investigating changes in fitness components that were not measured here (e.g. feeding efficiency or senescence).

The second possible explanation is dominance effects on the X-chromosome. It has been predicted that X-linked male-benefit/female-detriment alleles should preferentially be recessive in females, and female-benefit/male-detriment alleles should be dominant. Rice (1984) showed that both conditions allow high equilibrium frequencies of sexually antagonistic alleles. If most of the sexually antagonistic variation in the ancestral population was recessive in females, this could explain why there was no evidence of a reduction in female fitness (Figure 2a). The presence of a control X-chromosome in the MLX assay females would in this case masks both an increase in the frequency of recessive male-benefit alleles and a decrease in the frequency of dominant female-benefit alleles. These two explanations are not mutually exclusive, and both may have contributed to the lack of a decrease in female fitness. Although it is also possible that a signature of antagonism evolved between the time when the fitness data were collected (generation 40) and the RNA extractions were carried out (generation 50), we do not consider this explanation particularly likely since the other sex-limited evolution studies discussed above have detected signatures of antagonism after less than 30 generations (Morrow et al., 2008; Prasad et al., 2007; Rice, 1992). Finally, we cannot exclude the possibility that X-linked loci have a smaller contribution to female fitness compared to male fitness, although a previous study

of the ancestral population suggests that any such differences are modest at best (Gibson et al., 2002).

4.3 | Genomic location and function of genes that changed in expression

Genes that responded to the selection treatment were distributed throughout the genome (Table 1), with only around 80 of the transcripts that were identified as differentially expressed located on the X-chromosome. This suggests that most of the changes that occurred are in genes that are regulated by X-linked loci, a handful of which might be sufficient to drive the observed downstream changes. This is generally consistent with other results that suggest that sexually antagonistic loci may be few but of relatively large effect (Barson et al., 2015; Rice, 1992; Ruzicka et al., 2019). Interestingly, down-regulated transcripts in MLX compared to control populations were preferentially located on the X (Table 1). This is consistent with theory and previous empirical data that suggest that the X should be feminized (or at least demasculinized) compared to the autosomes (Long et al., 2012; Parisi et al., 2003; Rice, 1984; Sturgill, Zhang, Parisi, & Oliver, 2007). The highly significant overrepresentation of up-regulated transcripts on chromosome 4 (Table 1) in MLX compared to control populations may or may not be phenotypically relevant. Because chromosome 4 has very low rates of recombination, it is sufficient that a single gene which interacts with the X-chromosome be selected for increased expression in order to cause a correlated response across many genes on chromosome 4. Nevertheless, chromosome 4 has been proposed to be the remnant of an old sex chromosome in *Drosophila* (Vicoso & Bachtrog, 2013), so interactions between chromosome 4 and the X-chromosome could therefore be functionally important. In addition, chromosome 4 has been shown to be disproportionately important in determining viability (Charlesworth, 2015; Kenyon, 1967), so changes in expression on chromosome 4 could be the mechanism behind the apparent increase in male survival seen here as a result of male-limited selection (Figures S2 and S3). Finally, much of this overrepresentation may also be related to mito-nuclear conflict, since 6 of the 16 up-regulated genes located on chromosome 4 are previously characterized as mito-sensitive (i.e. influence male fitness depending on mitochondrial genotype).

The transcripts that changed in expression as a result of the selection treatment were in some cases consistent with previous phenotypic data, but were unexpected in other cases and suggest avenues for future exploration. A common theme within the overrepresented GO terms for up-regulated genes in MLX was metabolism (Table S5). This is interesting because the direction of the change is consistent with increased adult activity levels (Figure 2b), which has previously been shown to be a sexually antagonistic trait (Long & Rice, 2007), but is in the opposite direction to what we would expect from sexual dimorphism (males have a lower metabolic rate than females; Van Voorhies, Khazaeli, & Curtsinger, 2004). However, this pattern could also be related to mito-nuclear conflict, since mitochondrial genes preferentially accumulate male-deleterious alleles due to their female-limited transmission (Frank & Hurst, 1996; Innocenti et al., 2011). The X-chromosome has a reduced number of mito-sensitive genes compared to the autosomes in *Drosophila melanogaster*, suggesting the X is indeed a bad location for mito-nuclear genes because selection against male-deleterious alleles can only occur via their indirect effect of reduced inheritance via the matriline (Rogell et al., 2014). However, mito-sensitive genes are also overrepresented among genes important for male fitness (Rogell et al., 2014), so by decoupling inheritance of the X and the mitochondria, our selection treatment may have allowed more efficient selection against male-deleterious mito-associated alleles. Consistent with this, we found that both mito-annotated (Rogell et al., 2014) and mito-sensitive (Innocenti et al., 2011) genes were up-regulated more often than expected in the selected populations. There were also a number of terms associated with locomotory activity or muscle development, and testis-specific genes were overrepresented among up-regulated genes, so another nonexclusive explanation could be that up-regulation of metabolism is a result of increased overall activity levels or selection for improved performance in sperm competition. Other potentially interesting GO terms are discussed in the Supplementary Information.

4.4 | Sexual antagonism in gene expression and the evolution of sexual dimorphism

In contrast to results from phenotypic data, expression data showed a much stronger signature of sexual antagonism and were consistent with expectations from extant sexual dimorphism. Genes that changed significantly in expression did so overwhelmingly in the same direction as extant sexual dimorphism (Figure 2)—that is genes that were already up-regulated in males increased in expression in both sexes in the selected populations and genes that were down-regulated in males decreased in expression in both sexes in the selected populations—even though the magnitude of the change was small and somewhat smaller in females than in males. In addition, genes previously identified as significantly male-biased in expression were overrepresented among up-regulated transcripts, and female-biased genes were overrepresented among down-regulated transcripts (Table 3), despite the fact that male-biased genes are generally underrepresented on the X-chromosome (Parisi et al., 2003). This overrepresentation of male-biased genes probably explains the smaller change in expression in females compared to males, but female-specific adaption of the autosomes during the selection process may also play a role. These results are consistent with the prediction that sexual dimorphism is often a signature of fully or partially resolved sexual antagonism, even though the two need not always coincide (Cox & Calsbeek, 2009; Innocenti & Morrow, 2010). It also suggests that if X-linked expression could be fully decoupled between the sexes, then this would lead to an overall increase in the degree of sexual dimorphism.

Interestingly, there was a signature of antagonism among up-regulated genes, since there was a strong relationship between previous characterization as being sexually antagonistic (Innocenti & Morrow, 2010) and up-regulation after the selection treatment (Table 2). A caveat here is the fact that cryptic population substructure has since been found in the data set used to determine the sexually antagonistic nature of these loci (M. Reuter, personal communication), and this may have inflated the signature of antagonism in Innocenti and Morrow (2010). Nevertheless, an overrepresentation of such loci among up-regulated genes is consistent with our a priori predictions. There was no evidence of overrepresentation of loci more recently detected as sexually antagonistic (Ruzicka et al., 2019), but many of these loci were inferred to be coding changes unlikely to affect expression and may therefore not be detectable within our data set.

Increased sexual conflict has been suggested to induce an overall shift towards the male expression optimum (Hollis et al., 2014; Immonen et al., 2014; Innocenti et al., 2014; Perry et al., 2016) and might therefore be expected to parallel the changes seen here as a result of reducing female-specific selection and resolving sexual antagonism (but see Veltsos et al., 2017). Evidence for parallel changes was equivocal. There was no significant overrepresentation of genes identified by Innocenti et al. (2014), but there was overrepresentation of genes identified by Immonen et al. (2014). Given that changes as a result of alterations of sexual conflict seem to preferentially involve sex-biased genes (Hollis et al., 2014; Immonen et al., 2014; Innocenti et al., 2014), but that the direction of the change is sometimes opposite to the direction of sex bias (Parker et al., 2019; Veltsos et al., 2017), it seems likely that any overlap with sexually antagonistic loci is caused by the fact that both phenomena are related to the degree of sexual dimorphism and not because increased sexual conflict resolves antagonism per se (i.e. stronger selection under sexual conflict does not inevitably lead to genetic decoupling of male and female traits).

Resolution of sexual antagonism on the X may partly be mediated by dosage compensation, since there was a positive relationship between degree of up-regulation and distance from high-affinity sites (HAS), which are associated with dosage compensation (Figure 2d). It is known from cross-species comparisons that high-expression male-biased genes are more often located on the autosomes, whereas low-expression male-biased genes are more often located on the X, and that most X-linked male-biased genes are located outside of dosage compensation regions (Bachtrog et al., 2010). The positive association seen here is consistent with previous results demonstrating that dosage compensation is a constraint for male-biased genes, although with the data at hand we cannot determine the direction of causality—it could be that genes located farther from HAS changed more simply because they are more likely to be male-biased, not because genes located close to HAS were constrained in their response. However, this result is also interesting because it suggests that there is standing genetic variation for the degree or consistency of dosage compensation, something that bears further investigation.

There has been some discussion and conflicting results reported as to whether the X should be feminized (enriched in female-biased genes compared to the autosomes; e.g. Parisi et al., 2003), demasculinized (impoverished for male-biased genes compared to the autosomes; e.g. Sturgill et al., 2007), both (reviewed in Dean & Mank, 2014) or even masculinized (Patten, 2018). Which pattern is most prevalent may depend not only on dominance but also the nature of sex-specific mutational effects (Frank & Patten, 2020). Another complicating factor seems to be timescale. The X-chromosome often accumulates mutations more quickly than the autosomes due to more efficient selection of beneficial mutations and/or drift, a phenomenon known as the faster-X effect. Since testis-specific genes have generally been shown to be rapidly evolving, a larger proportion of these rapidly accumulating mutations may be X-linked in origin. Indeed, Zhang et al. (2010) found that young male-biased genes were enriched on the X in *Drosophila*, but that old male-biased genes were enriched on the autosomes, consistent with the idea that the X contributes to rapid evolutionary change, but that it is an unfavourable location for male-biased genes. The microevolutionary effects seen in this study are consistent with these macroevolutionary patterns—when selection in females was removed, X-linked female-biased/female-benefit and male-detriment genes were down-regulated. However by the same token, there were almost twice as many genes with significant upregulation compared to down-regulation (342 versus 176). This is consistent with the idea that X-linked male-biased genes are more constrained by selection in females, than female-biased genes are constrained by selection in males, and that these X-linked male-biased genes show a larger response when selection in females is removed.

5 | CONCLUSIONS

By forcing the X-chromosome to only be expressed in males over many generations, we changed the selection pressures on the X to become similar to those experienced by the Y-chromosome. Releasing males from constraints arising from counter-selection in females is predicted to lead to specialization for male fitness and particularly to masculinization of phenotypes that normally experience sexually antagonistic selection. Indeed, we found evidence of masculinization primarily via up-regulation of male-benefit genes and down-regulation of X-linked female-benefit genes. In addition, we found evidence that female locomotory activity became masculinized, a trait that has previously been identified as sexually antagonistic. Changes in other traits not previously characterized as sexually antagonistic in this species, such as vision and learning/memory, suggest that these traits may be valuable to study further in this context in future. Interestingly, we could detect evidence of microevolutionary changes consistent with previously documented macroevolutionary patterns in sex chromosome evolution, such as up-regulation of male-biased genes and down-regulation of female-biased genes after a chromosome becomes male-limited (Wright et al., 2018), an increase in the expression of metabolic genes

related to mito-nuclear conflict (Rogell et al., 2014) and evidence that dosage compensation effects can be altered (Bachtrog et al., 2010). These results confirm the importance of the X in the evolution of sexual dimorphism and as a source for sexually antagonistic genetic variation and demonstrate that experimental evolution can be a fruitful method for testing theories of sex chromosome evolution. Since previous whole-genome male-limited experimental evolution studies have not included an analysis of gene expression, it would be particularly interesting to study in future whether male-limited autosomal evolution produces qualitatively different results compared to male-limited X-chromosome evolution.

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AUTHOR CONTRIBUTIONS

JKA, AKC and EHM all contributed to designing the study. JKA collected and analysed the data, and wrote the manuscript with input from AKC and EHM.

DATA AVAILABILITY STATEMENT

Data are archived in the Gene Expression Omnibus under accession number GSE123827. https://doi.org/10.5061/dryad.wdbrv15k3.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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