ARTICLE (Discoveries section)

The effects of sex-biased gene expression and X-linkage on rates of sequence evolution in *Drosophila*

Authors: José Luis Campos[1], Keira Johnston[2], Brian Charlesworth[1]

[1] Institute of Evolutionary Biology, School of Biological Sciences, University of Edinburgh, Charlotte Auerbach Road, Edinburgh EH9 3FL, United Kingdom .

[2] Institute of Health and Wellbeing, University of Glasgow, 1 Lilybank Gardens, Glasgow G12 8RZ United Kingdom.

Corresponding author: José Luis Campos (j.campos@ed.ac.uk)

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Abstract

A faster rate of adaptive evolution of X-linked genes compared with autosomal genes (the faster-X effect) can be caused by the fixation of recessive or partially recessive advantageous mutations. This effect should be largest for advantageous mutations that affect only male fitness, and least for mutations that affect only female fitness. We tested these predictions in *Drosophila melanogaster* by using coding and functionally significant non-coding sequences of genes with different levels of sex-biased expression. Consistent with theory, nonsynonymous substitutions in most male-biased and unbiased genes show faster adaptive evolution on the X. However, genes with very low recombination rates do not show such an effect, possibly as a consequence of Hill-Robertson interference. Contrary to expectation, there was a substantial faster-X effect for female-biased genes. After correcting for recombination rate differences, however, female-biased genes did not show a faster X-effect. Similar analyses of non-coding UTRs and long introns showed a faster-X effect for all groups of genes, other than introns of female-biased genes. Given the strong evidence that deleterious mutations are mostly recessive or partially recessive, we would expect a slower rate of evolution of X-linked genes for slightly deleterious mutations that become fixed by genetic drift. Surprisingly, we found little evidence for this after correcting for recombination rate, implying that weakly deleterious mutations are mostly close to being semidominant. This is consistent with evidence from polymorphism data, which we use to test how models of selection that assume semidominance with no sex-specific fitness effects may bias estimates of purifying selection.

Introduction

The differences between the modes of inheritance of genes on the X chromosome and the autosomes are expected to affect their patterns of variation and evolution (reviewed by Meisel and Connallon 2013). Under suitable conditions with respect to the levels of dominance of new mutations, sex differences in selection coefficients, recombination rates and effective population size, theory shows that X-linked genes can evolve under positive selection more rapidly than autosomal genes – the so-called 'faster-X effect'. This reflects the fact that the hemizygosity of the X chromosome in males (in species with male heterogamety) means that positive selection can sometimes be more effective

on new X-linked mutations than on autosomal mutations with equivalent effects on fitness (Haldane 1924; Charlesworth et al. 1987; Meisel and Connallon 2013). This is, however, only likely to happen when evolutionary change depends on new mutations; fixation of variants present as standing variation tends to favour a slower rate of evolution of X-linked genes (Charlesworth et al. 1987; Orr and Betancourt 2001; Meisel and Connallon 2013). An important consequence of the faster-X effect is that X (or Z) chromosomes are likely to play a disproportionate role in speciation and evolutionary divergence (Orr and Coyne 1989; Presgraves 2008).

Faster-X evolution from new mutations is facilitated by partial recessivity of beneficial mutations (dominance coefficients less than one-half) (Charlesworth et al. 1987); and by a higher ratio of the effective population size of X-linked to autosomal genes (N_{eX}/N_{eA}) than the value of three-quarters expected under the standard Wright-Fisher model, which assumes equal variances in reproductive success in males and females (Vicoso and Charlesworth 2009; Meisel and Connallon 2013). Genes that only affect male fitness are more likely to exhibit a faster-X effect than genes that affect the fitnesses for both sexes. In contrast, genes that affect only female fitness will not experience a faster-X effect unless $N_{eX}/N_{eA} > ¾$ (Charlesworth et al. 1987; Vicoso and Charlesworth 2009; Mank et al. 2010; Meisel and Connallon 2013). With sexually antagonistic effects of mutations on fitness, a faster-X effect can arise with recessive or partially recessive mutations that increase male fitness at the expense of female fitness, or dominant or partially dominant mutations that increase female fitness at the expense of male fitness (Rice 1984; Charlesworth et al. 1987).

These theoretical expectations are supported by recent studies of *D. melanogaster* and its close relatives, using whole genome sequence comparisons between species and resequencing of multiple individuals of *D. melanogaster*. These studies showed that there is a faster rate of protein sequence evolution on the X chromosome (X) than on the autosomes (A), and that this is attributable to a greater incidence of fixations of selectively favourable nonsynonymous mutations on X compared with A (Langley et al. 2012; Mackay et al. 2012; Hu et al. 2013; Campos et al. 2014; Ávila et al. 2015).

In a previous study (Ávila et al. 2015), we tested the above predictions of the theory concerning sex-specific fitness effects by using genes with different levels of sex-biased expression (unbiased, male- and female-biased) in *Drosophila melanogaster*, and estimating the extent of adaptive evolution of non-synonymous mutations. We detected a faster-X effect for all categories of gene expression, including female-biased genes. The sex bias in gene expression for each gene was used as a proxy for male/female fitness, assuming that unbiased genes are likely to have similar fitness effects on both sexes (or to be sexually antagonistic), male-biased genes to affect male fitness more than female fitness, and vice-versa for female-biased genes. A faster-X effect was found for female-biased genes, even after adjusting for the effects of differences in recombination rate between X and A that reflects differences in N_e caused by selection at linked sites; such effects were detected by Campos et al. (2013) (see their Figure 2). However, Ávila et al. (2015) used a meta-analysis of *Drosophila* gene expression, most of which included whole-fly expression data. A FDR cut-off of 20% was used to classify genes as sex-biased. With this liberal criterion, 'female-biased' X-linked genes could still have significant expression levels in males, and hence be exposed to positive selection on the hemizygous X. Here we use a much more stringent criterion for sex biased gene expression, to ensure that female-biased genes can have little effect on male fitness.

Faster-X divergence for gene expression has been reported in Drosophila (Kayserili et al. 2012; Meisel et al. 2012), possibly driven by adaptive and recessive cis-regulatory mutations that affect gene expression levels (Coolon et al. 2015). A theoretical framework for how adaptive cis-regulatory mutations could be recessive was proposed by Meisel et al. (2012). Previous work has shown that many non-coding regions are functional and experience both negative and positive selection (Haddrill et al. 2005; Andolfatto 2005; Halligan and Keightley 2006; Halligan et al. 2013). In addition, a faster-X effect for non-coding regions has been described in *Drosophila* (Begun et al. 2007; Mackay et al. 2012; Hu et al. 2013), which could be related to the faster X-effect on gene expression. We have therefore extended the Ávila et al. (2015) analysis to non-coding regions, including 5′ UTRs, 3′ UTRs and long introns (introns shorter than 100bp appear to experience little selective constraint [Halligan and Keightley 2006; Parsch et al. 2010]).

We also tested for the existence of a slower-X effect for deleterious mutations. Much evidence suggests that mutations are partially recessive in their effects on fitness (Dobzhansky and Wright 1941; Muller 1950; Crow and Simmons 1983; García-Dorado and Caballero 2000; Manna et al. 2011). Purifying selection is thus usually expected to be stronger on the X than A, and a lower rate of fixation of slightly deleterious mutations is expected for X than for A, unless the X/A ratio of N_e is < ¾ (Mank et al. 2009; Vicoso and Charlesworth 2009). However, there is only weak empirical evidence for a slower-X (or Z) effect (Carneiro et al. 2012; Hvilsom et al. 2012; Veeramah et al. 2014; Rousselle et al. 2016). We tested for this effect by estimating the proportion of slightly deleterious mutations fixed on X and A in a comparison of a pair of closely related species. Overall, we did not find any substantial differences between X and A, suggesting that slightly deleterious mutations may be close to being semidominant. This was consistent with the results of comparisons between X and A of nucleotide site diversities at functional sites.

Results

Rates of adaptive evolution on X versus A

A total of 7853 *D. melanogaster* genes was available for this study (see Supplementary Table 1). As described in the Material and Methods, we used a dataset on germline gene expression to classify genes as male-biased, female-biased and unbiased genes, with a much more stringent criterion for sex-biased expression than that of Ávila et al. (2015). For this dataset, the largest group was that for unbiased A genes (N = 4529), and the smallest group was that for female-biased X genes (N = 64). The distribution of the three categories of expression between A and X was significantly different ($\chi^2 = 23.37$, $P < 0.01$), with a slight excess of female-biased genes on the X (20.9%) and a deficit of unbiased (16.8%) and male-biased (12.9%) genes; based on the number of X-linked genes (1250/7853), approximately 16% of genes in a given class would be expected to be on the X chromosome. In order to minimize the possibility that a gene that shows biased gene expression in the germline of one sex may be expressed in the soma of the opposite sex, we also used gene expression levels in heads to quantify sex specificity even more stringently, as described in the Material and Methods. After doing this, our sex-biased dataset was considerably smaller, with only 39 female-biased genes, but the distribution of genes among X and A was almost identical to that for the germline

dataset (unbiased, 16.3% on the X; male-biased, 12.9% on the X; female-biased, 20.8% on the X).

For all three categories in the germline-only dataset (unbiased, male- and female-biased), genes on the X showed a higher rate of adaptive nonsynonymous site evolution as measured by ω_a, the rate of substitution of positively selected mutations for a given class of functional sites relative to that for synonymous mutations (Figure 1; Supplementary Table 1). UTRs and long introns also showed a faster-X effect, although this was not significant for female-biased genes in long introns (Figure 1; Supplementary Table 1).

Use of the more conservative dataset that used expression data on heads gave results that were similar to those for the germline-only datasets, with the sole exception of female-biased 3′ UTRs (Supplementary Table 2). Overall, the results of Ávila et al. (2015) were confirmed by the present analysis, suggesting that factors other than dominance level may be involved in the faster-X effect, at least for female-biased genes. As seen in previous studies (Baines et al. 2008; Parsch and Ellegren 2013; Ávila et al. 2014; Kousathanas et al. 2014; Ávila et al. 2015), male-biased genes have the fastest rates of adaptive evolution, for both X and A (Figure 1; Supplementary Tables 1 and 2).

Factors potentially influencing the faster-X effect

Narrower breadth and lower levels of gene expression are known to be associated with increased rates of sequence evolution (Larracuente et al. 2008; Meisel et al. 2012). Using the criteria described in the Material and Methods, we found that, overall, X genes had a narrower breadth of gene expression than A genes ($P = 0.02$). This suggests that ω_a could be higher for the X because of a difference in breadth of gene expression. However, we observed no significant differences in expression breadth between X and A within the three pairs of groups of interest (unbiased, male-biased and female-biased genes) (Supplementary Table 3). We also found no difference in average expression levels between X and A ($P = 0.58$). When we compared X and A within the three pairs of groups of interest, we again found no significant differences (Supplementary Table 3). Thus, neither breadth of expression nor average expression seem to be involved in the higher rate of adaptive evolution of female-biased genes for X compared with A.

The rate of adaptive sequence evolution is also known to be positively correlated with the rate of crossing over per Megabase (Campos et al. 2014; Charlesworth and Campos 2014; Castellano et al. 2016). As was found in previous studies (Comeron et al. 2012; Campos et al. 2013), the X chromosome had a significantly higher effective rate of crossing over than the autosomes: mean for A = 1.14 cM/Mb, with 95% confidence interval (CI) (1.13, 1.16), and mean for X = 1.96 cM/Mb (CI: 1.86, 1.95) (this statistic corrects for the differential transmission of X and A through females and males: see Material and Methods for details). Campos et al. (2013) showed that N_{eX}/N_{eA} (estimated from the X/A ratio of 4-fold degenerate site diversities) for X and A genes with comparable effective recombination rates was close to ¾, except for genes in regions with very low rates of crossing over. Comparisons of X and A genes in regions with similar effective recombination rates should therefore largely remove any effects of departures of N_{eX}/N_{eA} from ¾, which might cause a faster-X effect for female-biased genes (see the introduction). To adjust for differences in effective recombination rates between X and A, we divided the genes for both X and A into four bins of recombination rates, using the germline dataset, because it had a larger sample size for male- and female- biased genes; as shown above, the initial results were similar for both datasets.

The effective recombination rates for A and X genes did not overlap for rates above ~2 cM/Mb, so that only the first two bins for X overlap those for A (see Figure 2). For nonsynonymous mutations, the confidence intervals for ω_a for the bins of unbiased and male-biased X genes with the lowest recombination rate overlapped those for A (Figure 2; Supplementary Table 4). However, the remaining X bins had significantly higher ω_a values than the corresponding A bins, including the second X bin with a similar effective recombination rate to the fourth bin of autosomal genes (proportion of ω_a values greater on the A: $P = 0$ and $P = 0.016$ for unbiased and male-biased genes, respectively). In contrast, female-biased genes (F) with similar effective recombination rates did not show a faster-X effect (Figure 2; Supplementary Table 4; 2nd A bin vs 1st X bin, $P = 0.349$; 4th A bin vs 2nd X bin, $P = 0.92$). Due to the small sample size of the first two XF bins that overlap in recombination with AF, we also grouped them into a single bin (21 genes); however, there was no significant difference from the whole AF dataset ($P = 0.622$, $\omega_{a\,XF} = 0.057$ vs. $\omega_{a\,\mathrm{AF}} = 0.063$).

We carried out similar analyses for non-coding sequences (Figure 2; Supplementary Table 4). For unbiased and male-biased genes, the patterns for UTRs and long introns were similar to those for nonsynonymous sites, such that ω_a for the X bin with the lowest recombination rate did not differ significantly from that for A genes with similar recombination rates ($P > 0.05$). The remaining bins for UTRs and long introns of unbiased and male-biased genes showed a faster-X effect, including the 2nd X bin that overlaps in recombination rate with the 4th A bin ($P = 0$). For UTRs of female-biased genes, there was a faster-X effect across all bins ($P < 0.05$). This result was stronger for 5′ UTRs (Supplementary Figure 1; 2nd A bin – 1st X bin, $P = 0.056$; 4th A bin – 2nd X bin, $P = 0.008$) but was also seen with 3′ UTRs (Supplementary Figure 1; 2nd A bin – 1st X bin, $P = 0.014$; 4th A bin – 2nd X bin, $P = 0.179$). For female-biased genes, there was a faster-X effect only for long introns in bins with a higher effective recombination rate on the X (Figure 2; 2nd A bin – 1st X bin, $P = 0.91$; note that, due to the small sample size, there is only one X bin that overlaps with A in recombination rate).

Purifying selection on X and A

We also performed several tests to compare the strength of purifying selection on mutations in X and A genes, since the exposure of mutations to selection in the hemizygous state in males is expected to result in larger selection coefficients for rare, deleterious X-linked mutations relative to comparable autosomal mutations, with the exception of mutations with male-limited effects and a dominance coefficient $h \geq 2/3$, as shown in the overview of relevant population genetics theory in the Supplementary Material. The theoretical results imply that parameters such as the rates of fixation of weakly deleterious mutations, and the ratios of diversity levels at sites under selection to those at neutral sites, are dependent on the product of the strength of selection and the effective population size (N_e). The interpretation of the measures described below is therefore complicated by possible differences in N_e between X and A, especially as N_e may be affected by hitchhiking and hence by the rate of recombination for the genomic region in question, as discussed above (see also Vicoso and Charlesworth 2009; Charlesworth and Campos 2014).

The first measure of the efficacy of purifying selection that we used was the estimate of the rate of non-adaptive substitutions at functional sites, relative to the rate

for synonymous substitutions (ω_{na}). For nonsynonymous sites, this was consistently lower for X than for A for all expression categories (Figure 3, Supplementary Table 5), suggesting a larger product of effective population size (N_e) and strength of selection against slightly deleterious mutations for X compared with A. The X – A differences were significant for the combined UTR dataset for all categories of gene expression, as well as for 5′ UTRs, 3′ UTRs and introns of unbiased genes, for 5′ UTRs of female-biased genes, and for 3′UTRs of male-biased genes.

However ω_{na} makes several assumptions that could affect the results (see the Discussion), especially the use of a model of selection that assumes semidominance with no sex-specific fitness effects (Eyre-Walker and Keightley 2009). We therefore used diversity statistics to estimate the efficacy of purifying selection; where appropriate, we applied correction factors to take into account possible differences in N_e between the X and A (see the theoretical section of the Supplementary Information). For the diversity data for the chromosomes as a whole, except for very low recombination regions, our previous work showed that the effective population size for the X chromosome, N_{eX}, is approximately equal to that for the autosomes, N_{eA}, when X and A regions with similar effective recombination rates are compared (Campos et al. 2013). For semidominant mutations with $h = ½$ and sufficiently strong selection ($N_e s >> 1$), diversity for functional sites on the X (π_F) is expected to be approximately three-quarters of that for A, assuming that these mutations are sufficiently strongly selected that $N_e s >> 1$; it will be less than this for partially recessive unbiased genes and male-biased genes. When $N_{eX} = N_{eA}$, the same should apply to the ratio of the mean pairwise nucleotide site diversity at putatively functional sites to the mean synonymous site diversity (π_F/π_S), assuming the latter to be close to neutral; this statistic has the advantage of reducing variability among different classes of genes caused by differences in their mutation rates.

Tables 1 and 2 show the mean values and 95% bootstrap confidence intervals for π_F and π_F/π_S, respectively, for each type of sequence and chromosome. For nonsynonymous sites, especially for π_F/π_S for unbiased genes, there is a clear indication of stronger selective constraints on X chromosomal genes; the same pattern is also noticeable for UTRs and introns in unbiased genes. When we applied a correction factor of 4/3 to the X, π_F and π_F/π_S for X were similar to, or larger than, the values for A

(Tables 1 and 2), so that there was no evidence for partial recessivity of deleterious mutations. As expected, π_F/π_S is larger for UTRs and introns than nonsynonymous sites. It is interesting to note that π_F/π_S for nonsynymous sites is larger for male-biased and female-biased than unbiased genes, paralleling what is seen for ω_{na} (Figure 3 and Supplementary Table 5), and suggesting that genes with sex biased expression have lower levels of selective constraints. This pattern is not seen for UTRs and introns.

We also examined how the higher overall effective recombination rate of the X might affect these statistics, by increasing N_e as a result of weaker hitchhiking effects. For all types of functional sequence, there was no difference in ω_{na} between X and A for regions that overlap in their effective recombination rates (Figure 4), suggesting that the difference in effective population sizes between X and A cancels the X-A differences in selection coefficients against new deleterious mutations (see the theoretical section of the Supplementary material). The same pattern was found for π_F/π_S (Supplementary Figure 2 and Supplementary Table 4). Since our previous work showed that the ratio N_{eX}/N_{eA} was approximately ¾ in overlap regions other than those with low recombination rates (Campos et al. 2013), we did not use a correction factor for π_F/π_S for the overlap regions studied here, because this difference in N_e exactly cancels the difference in strength of selection on the hypothesis of semidominance. But after applying the 4/3 correction, π_F for the overlap regions was either higher for X than for A or similar to A (Supplementary Figure 3). Again, there was no evidence for significant departures from semidominance for slightly deleterious mutations.

Discussion

Under suitable conditions on the levels of dominance of the fitness effects of beneficial mutations, there should be a faster rate of fixation of new mutations on the X chromosome than the autosomes – the faster-X effect (Rice 1984; Charlesworth et al. 1987; Meisel and Connallon 2013). Genes expressed only in males are the most likely to show such an effect, whereas it should not be seen for genes expressed only in females, unless the effective population size for the X is higher than that for the autosomes. Consistent with these theoretical predictions, the results presented above show that substitutions in most types of functionally significant sequences in male-biased and unbiased genes (nonsynonymous sites, UTRs and long introns) tend to

exhibit faster rates of substitution of beneficial mutations for X genes compared with A genes (Figures 1 and 2).

However, our analyses also showed that genomic regions with very low rates of recombination do not show a faster-X effect (Figure 2 and Supplementary Table 4), consistent with previous observations that showed a lack of adaptive evolution at nonsynonymous sites in very low recombination regions of the *D. melanogaster* genome (Campos et al. 2014). This observation was interpreted in terms of strong Hill–Robertson interference in such regions (Hill and Robertson 1966; Felsenstein 1974), whereby both positive and negative selection at closely linked sites reduce the rate of fixation of beneficial mutations (Campos et al. 2014; Charlesworth and Campos 2014; Castellano et al. 2016).

The influence of recombination rate on adaptive evolution caused by Hill-Robertson interference is also probably responsible for the fact that the faster-X effect seen for nonsynonymous and long intron substitutions in female-biased genes disappears after correcting for their recombination rates – for these categories, only genes with a higher effective recombination rate on the X chromosome than the autosomes show a faster-X effect. Thus, the faster-X effect that we observed previously for nonsynonymous mutations in female-biased genes (Ávila et al. 2015) is probably due to an overall higher recombination rate for these genes, which reduces their exposure to Hill-Robertson interference.

As has been found previously for many different systems (Baines et al. 2008; Parsch and Ellegren 2013; Ávila et al. 2014; Kousathanas et al. 2014; Ávila et al. 2015), male-biased genes exhibited higher rates of adaptive evolution at nonsynonymous sites compared with unbiased and female-biased genes, for both X and A genes. Such an effect was not observed, however, for UTRs and long introns (Figure 1 and Supplementary Table 1). It is unclear why such a difference between types of sequence should occur, and it casts doubt on the frequently expressed idea that sexual selection causes faster rates of evolution of male-biased genes (Parsch and Ellegren 2013), since this is just as likely to affect regulatory mutations as amino-acid mutations. We also found evidence for weaker selective constraints on the protein sequences of genes with sex-biased expression, but not on UTRs or long introns.

The extent of between-species divergence in the level of gene expression has been also found to be greater for X than A in Drosophila (Kayserili et al. 2012; Llopart 2012; Meisel et al. 2012), suggesting faster-X adaptive evolution of cis-regulatory sequences (Kayserili et al. 2012; Meisel et al. 2012; Coolon et al. 2015). This hypothesis is consistent with the observation of faster-X divergence of non-coding sequences in Drosophila (Hu et al. 2013); that study did not, however, analyze the contribution of positively selected substitutions to differences between X and autosomes. Our results provide quantitative estimates of the extent of X - A differences in the rates of fixation due to positive selection (Figures 1 and 2; Supplementary Table 1), and demonstrate an adaptive faster-X effect for UTRs and long introns, consistent with the results of Begun et al. (2007) and Mackay et al. (2012). The faster-X effect for non-coding regions may well be related to the faster X-effect for gene expression. However, the faster-X effect that was found for the UTRs of female-biased genes (Figures 1 and 2; Supplementary Table 1) is not predicted by the standard theoretical models, and is not associated with a difference in recombination rate; furthermore, no faster-X effect for gene expression in genes with expression biased towards female-limited reproductive tissues was found by Meisel et al. (2012). Differences between X and A in traits other than male hemizygosity and recombination rate must be causing this pattern, possibly reflecting the specific biological functions of the small group of genes involved.

A much-debated question has been the relative contributions of protein-coding sequences versus non-coding sequences to adaptive evolution (e.g. Carroll 2000; Hoekstra and Coyne 2007; Stern and Orgogozo 2008). Our analyses showed that mutations in functional non-coding regions showed considerably higher rates of adaptive evolution than nonsynonymus mutations. These results are consistent with previous work on Drosophila using both McDonald-Kreitman type approaches (Andolfatto 2005; Begun et al. 2007; Mackay et al. 2012; Campos et al. 2017) and selective sweep signals (Elyashiv et al. 2016). One explanation is that non-coding regions are less constrained by purifying selection than nonsynonymous sites (ω_{na} is higher for non-coding regions than for nonsynonymous sites [Figures 3 and 4; Supplementary Table 5]), possibly because they are subject to fewer pleiotropic restrictions (Orr 1998; Carroll 2000; Orr 2000), making it easier for a new mutation to confer a selective advantage. These results are similar to the recent finding of higher

rates of positive selection observed in genes that are less constrained by purifying selection (Campos et al. 2017). Higher rates of adaptive sequence evolution in non-coding regions compared with protein sequences have been also found in other systems, such as mice (Halligan et al. 2013) and the flowering plant *Capsella* (Williamson et al. 2014).

We found evidence for differences in the strength of purifying selection between X and A, at least for unbiased genes, when examining the overall patterns of diversity for the two chromosomes (Tables 1 and 2). Such a pattern is expected for sufficiently strongly selected deleterious mutations ($N_e s >> 1$) with dominance coefficients $h \leq ½$ (see the theoretical section of the Supplementary Material). There is much evidence to suggest that h for deleterious mutations is mostly nonzero but < ½, i.e., mutations are partially recessive in their effects on fitness (Dobzhansky and Wright 1941; Muller 1950; Crow and Simmons 1983; García-Dorado and Caballero 2000; Manna et al. 2011). However, our analyses of diversities at functional sites, π_F, (Table 1; Supplementary Figure 3) and their ratios to synonymous site diversities, π_F/π_S, (Table 2; Supplementary Figure 2) showed no evidence that h was substantially less than ½.

The same conclusion was suggested by comparisons of ω_{na} between X and A (Figures 3 and 4; Supplementary Table 5). It is important to note, however, that the estimates of statistics such as ω_{na} and α (the proportion of between-species substitutions in a functional category that were fixed by positive selection) could be influenced by the dominance coefficients of deleterious mutations. The DFE-alpha method for estimating these quantities (Eyre-Walker and Keightley 2009) predicts the contribution of slightly deleterious mutations to variation and evolution using a population genetic model of autosomal loci subject to deleterious mutations with $h = ½$. This raises the question of the reliability of parameter estimates from DFE-alpha when h is < ½, for which the selection equations differ substantially from those for with semidominance, except for mutations with no fitness effects on males (see the theoretical section of the Supplementary Material). Using an approach similar to DFE-alpha, Veeramah et al. (2014) showed that, except for very low h values ($h < 0.2$), the dominance coefficient has only a small effect on the relative values of α estimates for X and A for human data (see Figure 2 of Veeramah et al. 2014). Their Figure 2 suggests that the assumption of $h = ½$, as was made here, should not lead to serious problems inferences concerning the

faster-X effect for adaptive mutations. This conclusion is strengthened by the analyses of purifying selection just described, which suggested that h is close to ½ for deleterious mutations (see Tables 1 and 2). Similarly, recessive effects of deleterious variants cannot explain the observed pattern of higher codon usage on the X (see Campos et al. 2013).

The comparisons between X and A of the corrected values of π_F and π_F/π_S (Tables 1 and 2) yielded many cases in which the X-A difference was significantly *larger* than that predicted on the hypothesis of strong selection with h = ½. This anomaly is larger for introns and UTRs. If there is a sizeable contribution from weakly selected mutations with $N_e s \leq 1$, the expected X/A ratio of π_F/π_S will be less than 4/3; in the limit of all mutations being neutral, it will approach 1 when $N_{eX} = N_{eA}$, as is the case for the two chromosomes as a whole. Multiplication by 4/3 would then be an overcorrection. Consistent with this hypothesis, inspection of the DFEs of the different sequence classes suggests that a considerably greater fraction of non-coding sequence mutations are weakly selected (15-26% mutations have $N_e s$ in the range 0 -1), than nonsynonymous sites (< 7% with $N_e s$ 0 - 1) – see Supplementary Table 6. Further theoretical predictions that take stochastic effects and the DFE parameters into account will be needed to test this hypothesis in detail.

Material and Methods

We used the same dataset as in Ávila et al. (2015), consisting of polymorphism data from 17 haploid genomes from the Gikongoro (Rwanda) population of *D. melanogaster* provided by the Drosophila Population Genomics Project 2 (Pool et al. 2012). As outgroup material, we used sequences from *D. yakuba* and *D. simulans* obtained from the *D. melanogaster–D. simulans–D. yakuba* gene alignments of Hu et al. (2013). The coding sequence data were filtered and analyzed as described in Campos et al. (2014). In brief, the 17 *D. melanogaster* genomes were obtained from the DPGP2 data set (http://www.dpgp.org/dpgp2/DPGP2.html). We mapped and aligned the reads to the reference sequence (r5.33, available on Flybase (http://flybase.org/) using BWA (Li and Durbin 2009). We used the Genome Analysis Toolkit (GATK), to do multisample SNP calling (DePristo et al. 2011). From the multisample VCF file, we made a consensus sequence FASTA file for each individual using a custom Perl script. Using the coding sequence coordinates, we extracted their sequences and made FASTA alignments

including the *D. yakuba* and *D. simulans* outgroups. We excluded 225 genes located in the autosomal heterochromatic regions and on chromosome four, where crossing over is absent (Campos et al. 2012; Campos et al. 2014).

We calculated pairwise nucleotide diversity (π, Tajima 1983) at putatively functional sites (π_F) and neutral synonymous site diversity (π_S). π_F / π_S was calculated as the ratio of the mean of π_F to the mean of π_S for the set of genes in question. As functional sites we considered nonsynonymous sites, 5′ and 3′ UTRs, and introns longer than 100 bp – shorter introns are under less constraint (Halligan and Keightley 2006). For the analyses of UTRs and introns, we masked any coding sequences and exons, respectively, and excluded any sequence with no outgroup information. To assign sites as synonymous and nonsynonymous and to estimate the nonsynonymous divergence and synonymous divergences, K_A and K_S, we used the method of Comeron (1995) – see Campos et al. 2014 for further details. To obtain the non-coding divergence data, we applied a Kimura two-parameter correction (Kimura 1980). We used the outgroup *D. yakuba* for coding sites and UTRs; and *D. simulans* for long introns.

Female crossing over rates for these genes, in terms of centiMorgans per megabase, were obtained from Comeron et al. (2012). These rates were multiplied by two-thirds for X-linked genes and by one-half for autosomal genes, respectively. This procedure provides estimates of 'effective recombination rates', taking into account the absence of recombination between homologous chromosomes in males, and the fact that X-linked genes and autosomal genes spend two-thirds and one-half of their time in females, respectively. This allows comparisons between X-linked and autosomal genes that experience the same rates of recombination as far as evolutionary processes are concerned (Campos et al. 2014).

In order to classify genes as unbiased, male- or female-biased, we used the tissue-specific expression values (in RPKM) of Brown et al. (2014). To obtain female expression values, we used female ovaries; for male expression values we used the average of gene expression in testes and accessory glands of male flies. In a first step, we obtained a sex bias ratio (SBR) value as: log 2 (male value / female value). We classified genes with a SBR > 1 as male-biased; genes with SBR < –1 as female-biased, and genes with SBR between –1 and 1 as unbiased. This is equivalent to using a 2-fold

expression difference between the sexes as the criterion for sex bias. In a second step, male and female expression values were ranked, and the 15th percentiles of their distributions were selected. If a gene was categorized as male-biased in the first step, and showed expression in females (ovary) of less than the female 15th percentile value, the gene was ultimately labelled as male-biased, and vice-versa for female bias. The aim of the second step was to ensure that biased genes have a very low level of expression in the opposite sex. Genes were then sorted into six categories, based on chromosomal location (autosomal vs. X-linked) and the sex-biased expression categories described above. Our final dataset consisted of 6603 autosomal genes and 1250 genes.

A potential reason for a faster-X effect for female-biased genes (which is contrary to theoretical predictions) is that such genes may exhibit some expression in males in somatic tissues, allowing positive selection to act when the gene is in a hemizygous state, even though the gene has been classed as female-biased according to the gonad-based sex bias ratio. To deal with this problem, we made a second dataset using gene expression in both heads and germline to define sex bias. To be classed as male-biased in expression, a gene must then meet two conditions. First, the gene must be expressed in the male gonad and head at a value greater than the male 15th percentile values for both the relevant tissues. Second, expression in the female ovary and head must be lower than the 15th percentile expression values for both tissues respectively; complementary criteria were used to classify a gene as female-biased.

The breadth of expression of a gene across different tissues is known to affect its rates of protein sequence evolution (Larracuente et al. 2008), and thus needs to be taken into account when comparing X and A. We measured this by τ, the proportion of tissues in which a gene is found to be expressed. τ ranges from 0 (for broadly expressed genes) to 1 (for narrowly expressed genes) (Yanai et al. 2005). Similarly, because levels of gene expression affect protein evolution (Larracuente et al. 2008), we calculated level of gene expression as the average expression across tissues for each gene.

We used DFE-alpha (Eyre-Walker and Keightley 2009) to estimate ω_a, the ratio of the rate of adaptive substitutions per basepair relative to neutral substitutions for nonsynonymous sites, UTR sites and sites in long introns. This method attempts to correct for the contribution of slightly deleterious mutations to polymorphism and

divergence and the impact of recent demographic change. We used *D. yakuba* ($K_S \approx$ 0.25) as an outgroup for CDS and UTRs, and *D. simulans* ($K_S \approx 0.12$) for long introns. We used a demographic model where the population at initial size N_1 (set to 100 in the DFE-alpha program) experienced a step change to N_2 at n generations in the past. A preliminary analysis for the whole dataset showed that a constant population size model gave a poor fit to the data, consistent with the results of Pool et al. (2012) that show that *D. melanogaster* African populations have apparently experienced a population expansion. To calculate confidence intervals we generated replicate bootstrap estimates for each group separately by resampling genes 1000 times within a given group, and running DFE-alpha for each bootstrap. In order to test for a significant difference in a given statistic between a pair of categories, such as X and A, we calculated the proportion of its bootstrap values that were larger for one category than the other.

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Table 1. π_F values for all expression categories and functional classes. π_F for the X shown in the fifth column was corrected by multiplying by 4/3. (95% bootstrap confidence intervals in brackets.)

Functional class	Expression bias	A	X	Corrected X value	*P*
Nonsynonymous	Unbiased	0.00122 (0.00118, 0.00126)	0.00109 (0.00101, 0.00118)	0.00145 (0.00135, 0.00157)	1
	Male	0.00161 (0.00153, 0.00171)	0.00161 (0.00139, 0.00181)	0.00214 (0.00185, 0.00242)	1
	Female	0.00171 (0.00153, 0.00192)	0.00159 (0.00121, 0.00203)	0.00212 (0.00161, 0.00271)	0.919
5′ and 3′ UTRs	Unbiased	0.00370 (0.00362, 0.00379)	0.00359 (0.00343, 0.00375)	0.00479 (0.00457, 0.00500)	1
	Male	0.00419 (0.00404, 0.00433)	0.00425 (0.00383, 0.00466)	0.00567 (0.00510, 0.00621)	1
	Female	0.00394 (0.00359, 0.00435)	0.00393 (0.00338, 0.00453)	0.00524 (0.00450, 0.00604)	0.999
5′ UTRs	Unbiased	0.00400 (0.00389, 0.00412)	0.00374 (0.00350, 0.00398)	0.00499 (0.00467, 0.00531)	1
	Male	0.00419 (0.00398, 0.00441)	0.00445 (0.00399, 0.00499)	0.00593 (0.00532, 0.00665)	1
	Female	0.00453 (0.00395, 0.00522)	0.00389 (0.00308, 0.00472)	0.00518 (0.00411, 0.00630)	0.849
3′ UTRs	Unbiased	0.00350 (0.00341, 0.00359)	0.00351 (0.00331, 0.00368)	0.00467 (0.00442, 0.00491)	1
	Male	0.00418 (0.00399, 0.00437)	0.00413 (0.00360, 0.00467)	0.00551 (0.00480, 0.00622)	1
	Female	0.00356 (0.00316, 0.00400)	0.00392 (0.00314, 0.00475)	0.00523 (0.00419, 0.00634)	0.995
Introns	Unbiased	0.00470 (0.00459, 0.00481)	0.00472 (0.00455, 0.00490)	0.00630 (0.00607, 0.00653)	1
	Male	0.00474 (0.00458, 0.00490)	0.00482 (0.00437, 0.00531)	0.00643 (0.00583, 0.00708)	1
	Female	0.00444 (0.00406, 0.00488)	0.00458 (0.00413, 0.00497)	0.00611 (0.00550, 0.00663)	1

P: Proportion of bootstraps in which the value of π_F for X (after multiplication by 4/3) was greater than that for A.

Table 2. π_F/π_S values for all categories and gene features. π_F/π_S for the X shown in the fifth column was corrected by multiplying by 4/3. (95% bootstrap confidence intervals in brackets.)

Functional class	Expression bias	A	X	Corrected X value	*P*
Nonsynonymous	Unbiased	0.0882 (0.0851, 0.0912)	0.0695 (0.0644, 0.0753)	0.0927 (0.0859, 0.1004)	0.866
	Male	0.0997 (0.0938, 0.1062)	0.0933 (0.0789, 0.1091)	0.1244 (0.1053, 0.1455)	0.997
	Female	0.1082 (0.0935, 0.1254)	0.0827 (0.0596, 0.1107)	0.1103 (0.0794, 0.1476)	0.52
5′ and 3′ UTR	Unbiased	0.2732 (0.2661, 0.2810)	0.2335 (0.2213, 0.2451)	0.3113 (0.2950, 0.3268)	1
	Male	0.2581 (0.2462, 0.2702)	0.2492 (0.2246, 0.2740)	0.3322 (0.2995, 0.3653)	1
	Female	0.2530 (0.2221, 0.2857)	0.2141 (0.1719, 0.2683)	0.2854 (0.2292, 0.3577)	0.8
5′ UTRs	Unbiased	0.2970 (0.2875, 0.3065)	0.2425 (0.2263, 0.2595)	0.3233 (0.3017, 0.3459)	0.985
	Male	0.2571 (0.2420, 0.2728)	0.2626 (0.2292, 0.2998)	0.3501 (0.3055, 0.3998)	1
	Female	0.2916 (0.2501, 0.3413)	0.2083 (0.1539, 0.2724)	0.2778 (0.2052, 0.3632)	0.384
3′ UTRs	Unbiased	0.2590 (0.2504, 0.2669)	0.2278 (0.2139, 0.2426)	0.3038 (0.2852, 0.3235)	1
	Male	0.2559 (0.2418, 0.2699)	0.2429 (0.2141, 0.2728)	0.3239 (0.2855, 0.3638)	0.999
	Female	0.2289 (0.1974, 0.2625)	0.2125 (0.1667, 0.2682)	0.2833 (0.2223, 0.3576)	0.915
Introns	Unbiased	0.3331 (0.3207, 0.3456)	0.3087 (0.2912, 0.3258)	0.4115 (0.3883, 0.4345)	1
	Male	0.2818 (0.2663, 0.2981)	0.2887 (0.2513, 0.3334)	0.3849 (0.3351, 0.4446)	1
	Female	0.2436 (0.2129, 0.2791)	0.2471 (0.2079, 0.3052)	0.3294 (0.2772, 0.4069)	0.997

P: Proportion of bootstraps in which the value of π_F for X (after multiplication by 4/3) was greater than that for A.

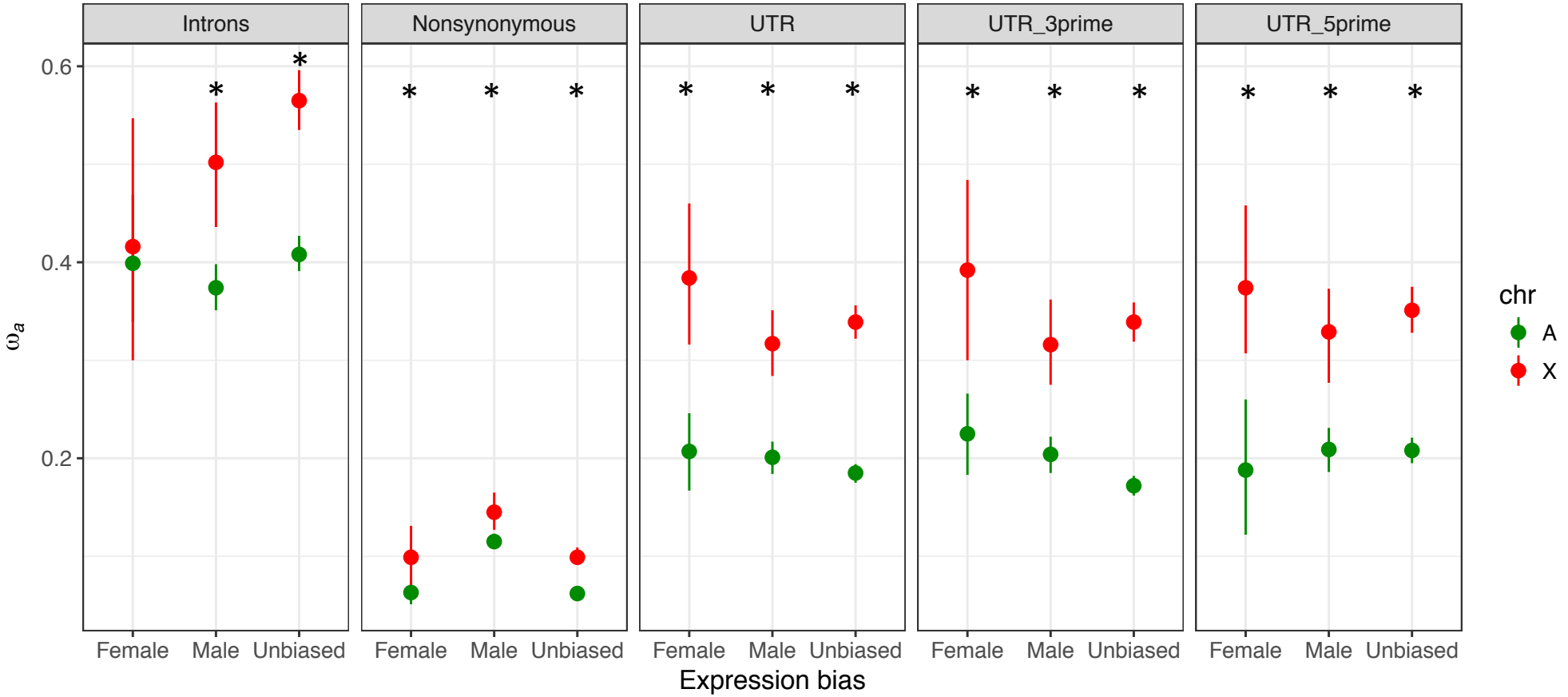
Figure legends

Figure 1. Plots of ω_a values for all categories and gene features, using germline expression levels to classify genes as unbiased, male-biased and female-biased (vertical bars represent 95% bootstrap confidence intervals). Asterisks show significant ($P < 0.05$) differences between A and X values, calculated as the proportion of bootstraps with larger ω_a value for A than X.

Figure 2. Plots of ω_a against the mean effective recombination rates for bins of genes, for the three categories of functional sequences. Confidence intervals were obtained by bootstrapping. Lines joining the bins are quadratic fits for the four ω_a values. Because of the small number of female-biased genes, only 3 bins were used for long introns.

Figure 3. Plots of ω_{na} values for all categories and gene features, using germline expression levels to classify genes as unbiased, male-biased and female-biased (vertical bars represent 95% bootstrap confidence intervals). Asterisks show significant ($P< 0.05$) differences between A and X values, calculated as the proportion of bootstraps with an ω_{na} value larger for X than A.

Figure 4. Plots of ω_{na} against the mean effective recombination rates for bins of genes, for the three categories of functional sequences. Confidence intervals were obtained by bootstrapping. Lines joining the bins are quadratic fits for the four ω_{na} values. Because of the low number of female-biased genes, only 3 bins were used for long introns.


Introns
Nonsynonymous
UTR
UTR_3prime
UTR_5prime
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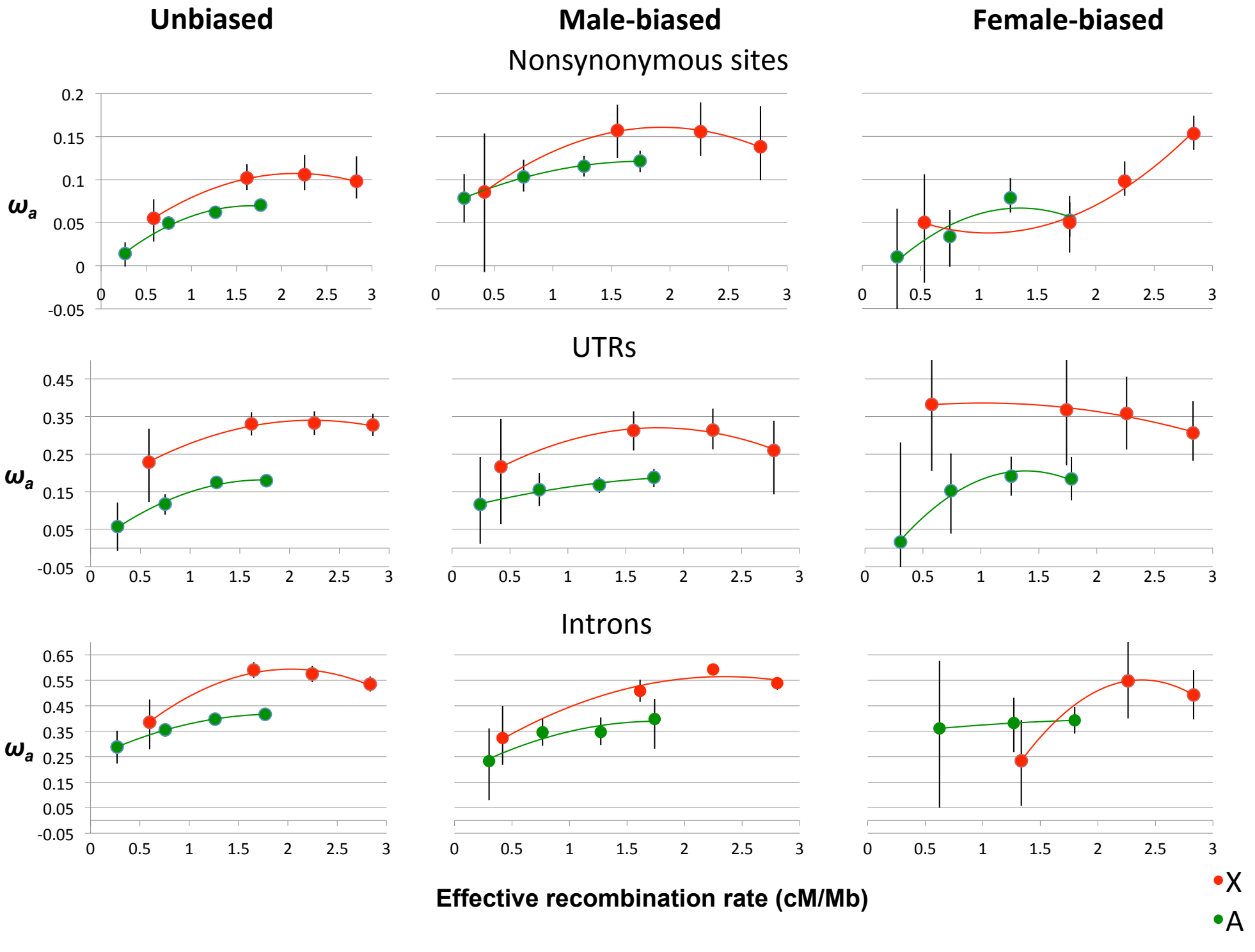
0.2
ω_a
Female
Male
Unbiased
Expression bias
chr
A
X


Unbiased
Male-biased
Female-biased
Nonsynonymous sites
UTRs
Introns
ω_a
0.2
0.15
0.1
0.05
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-0.05
0.45
0.35
0.25
0.15
0.05
-0.05
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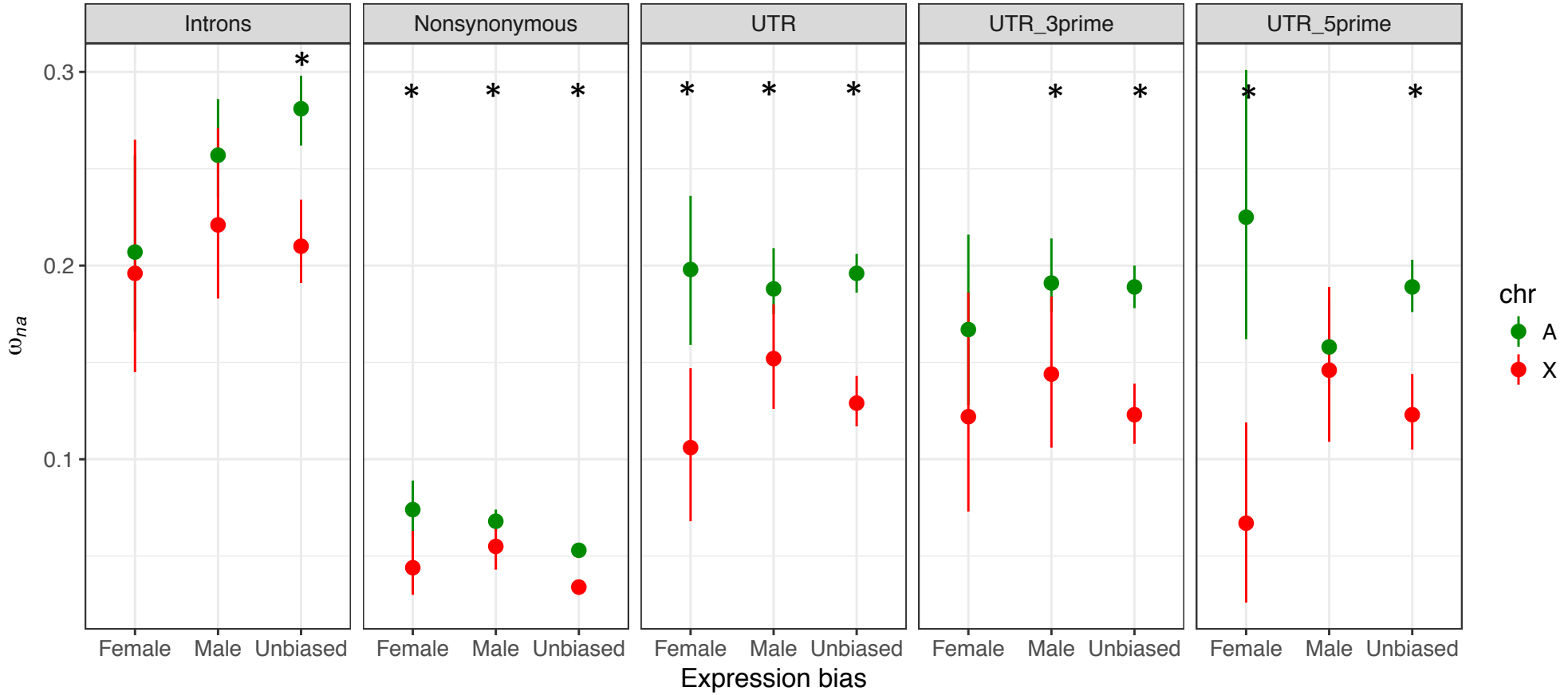
1
1.5
2
2.5
3
Effective recombination rate (cM/Mb)
X
A


Introns
Nonsynonymous
UTR
UTR_3prime
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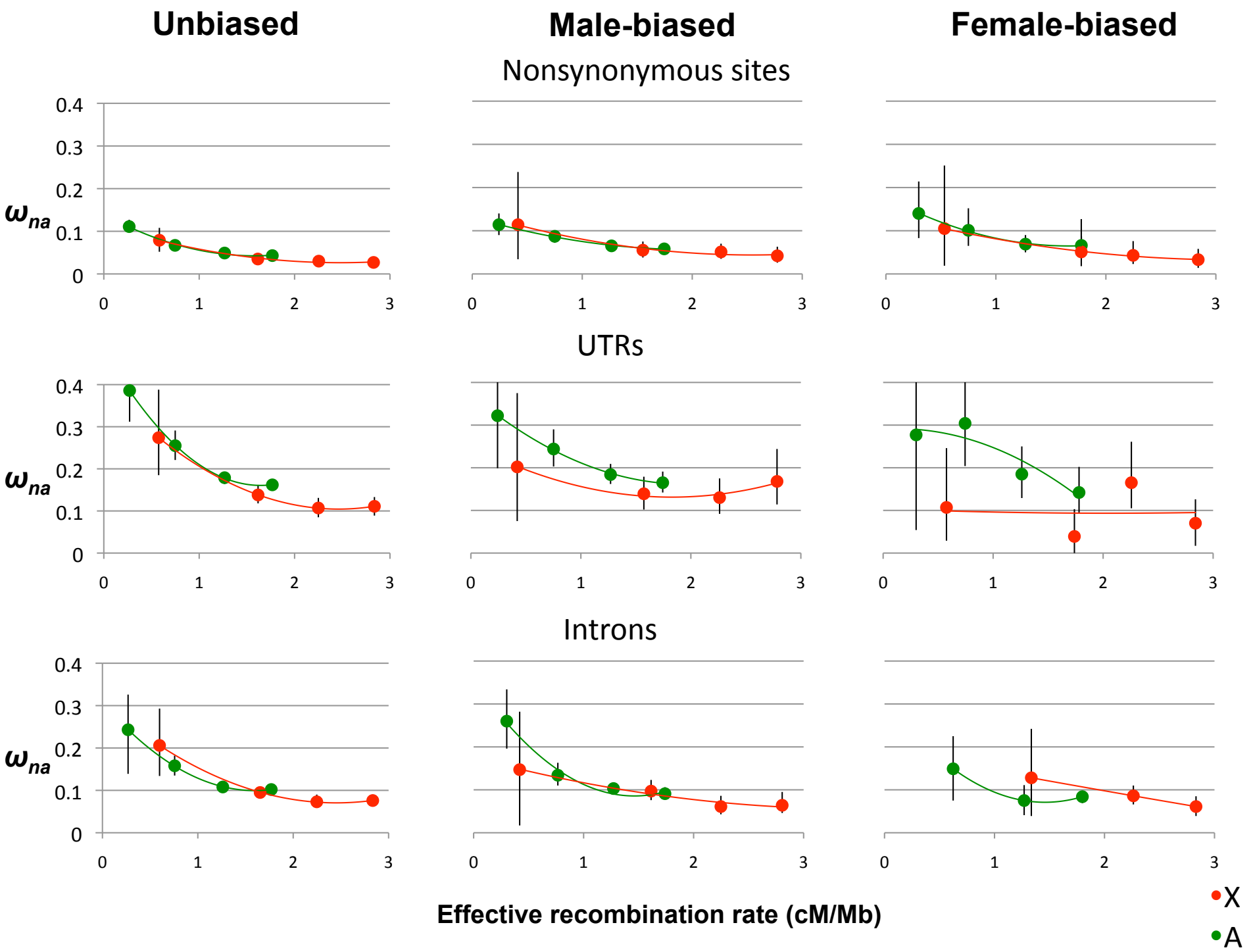
ω_{na}
Female
Male
Unbiased
Expression bias
chr
A
X


Unbiased
Male-biased
Female-biased
Nonsynonymous sites
UTRs
Introns
ω_{na}
0.4
0.3
0.2
0.1
0
0
1
2
3
Effective recombination rate (cM/Mb)
X
A