Sex differences in life span: Females homozygous for the X chromosome do not suffer the shorter life span predicted by the unguarded X hypothesis

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Abstract

Lifespan differs between the sexes in many species. Three hypotheses to explain this interesting pattern have been proposed, involving different drivers: sexual selection, asymmetrical inheritance of cytoplasmic genomes, and hemizyosity of the X(Z) chromosome (the unguarded X hypothesis). Of these, the unguarded X has received the least experimental attention. This hypothesis suggests that the heterogametic sex suffers a shortened lifespan because recessive deleterious alleles on its single X(Z) chromosome are expressed unconditionally. In *Drosophila melanogaster*, the X chromosome is unusually large (~20% of the genome), providing a powerful model for evaluating theories involving the X. Here, we test the unguarded X hypothesis by forcing *D. melanogaster* females from a laboratory population to express recessive X-linked alleles to the same degree as males, using females exclusively made homozygous for the X chromosome. We find no evidence for reduced lifespan or egg-to-adult viability due to X homozygosity. In contrast, males and females homozygous for an autosome both suffer similar, significant reductions in those traits. The logic of the unguarded X hypothesis is indisputable, but our results suggest that the degree to which recessive deleterious X-linked alleles depress performance in the heterogametic sex appears too small to explain general sex differences in lifespan.

Key words

Ageing, *Drosophila*, lifespan, sex differences, unguarded X, X chromosome

Introduction

Sex differences in lifespan are frequently observed in nature (Promislow 2003; Bonduriansky et al. 2008; Maklakov and Lummaa 2013; Austad and Fischer 2016). Most commonly, females live longer than males (Comfort 1979; Trivers 1985; Finch 1990; Promislow 1992), but the opposite also occurs (Promislow et al. 1992; Liker and Székely 2005). Three hypotheses explaining this interesting phenomenon have been suggested, based on sexual selection, asymmetrical inheritance of cytoplasmic genomes and sex chromosome linked recessive deleterious mutations. The sexual selection hypothesis stems from the fact that males and females often maximize fitness through different strategies. In many species, for instance, males are selected to display visually or sonically, or to engage in territorial fights. The function of these activities is to secure matings, but they also shorten expected lifespan by attracting predators and increasing the risk of injury (Williams 1957; Bonduriansky et al. 2008). Male competition over mating opportunities may also negatively impact female lifespan, either through intense harmful courtship or excessive mating (Svensson and Sheldon 1996; Promislow 2003; Bonduriansky et al. 2008; Adler and Bonduriansky 2014). Additionally, lifespan may depend on allocation of resources into somatic maintenance (Flatt 2011). Sex differences in lifespan can therefore arise when sexual selection is stronger in one sex (commonly males), favouring higher allocation of resources into early reproduction at the cost of somatic maintenance (Rolff 2002; Graves 2007; Bonduriansky et al. 2008; Archer et al. 2012).

A second hypothesis for the common nature of sex differences in lifespan is based on the fact that cytoplasmic genomes are exclusively inherited from mother to offspring (Frank and Hurst 1996; Gemmell et al. 2004). This asymmetrical pattern of inheritance causes selection on cytoplasmic genomes to operate exclusively in females, allowing male-detrimental mutations to accumulate through drift and selection when neutral or beneficial in females (Innocenti et al. 2011). Males may

thus carry a higher genetic load in mitochondrial genomes than females, with potentially negative consequences for male lifespan (Camus et al. 2012).

Finally, it has been suggested that sex differences in lifespan could result from the unequal copy numbers of sex chromosomes present in the heterogametic and homogametic sexes (the unguarded X hypothesis). In the homogametic sex, recessive X (Z)-linked deleterious alleles are normally masked by functional loci on the homologous X (Z) chromosome. In the heterogametic sex, however, most X (Z)-linked loci do not have a homologous copy on the Y (W) chromosome, which may cause this sex to suffer higher mortality rates (Trivers 1985). A similar argument can also be made for Y (W) specific genes. Thus, taken collectively, there are several mutually non-exclusive factors that potentially contribute to determining the relative lifespan of males and females. Any differences between the sexes in expected lifespan, generated by any of above mechanisms, could possibly be further augmented through accumulation of mutations with sex-specific or sex-biased effects on ageing (Medawar 1952; Williams 1957).

Of the three hypotheses for sex differences in lifespan, the unguarded X hypothesis has received the least attention. In support of the hypothesis, it has been found that the sex ratio across tetrapods is substantially biased towards the homogametic sex, but there are several competing hypotheses that could explain this pattern (Pipoly et al. 2015). A more explicit prediction made by the unguarded X hypothesis is that, during inbreeding, the homozygous sex should suffer a larger reduction in lifespan and in other traits under directional selection. This is because only the homogametic sex is affected by inbreeding of the X chromosome, while inbreeding of the autosomes affects both sexes. Two studies on inbreeding of the seed beetle *Callosobruchus maculatus* indeed found patterns in line with this prediction (Fox et al. 2006; Bilde et al. 2009).

However, since severe inbreeding actually extended male lifespan in a system where they are the heterogametic sex, an explanation based on sex-specific allocation patterns caused by sexual selection seems more plausible (Bilde et al. 2009). In *Drosophila melanogaster*, the X chromosome comprises ~20% of the genome, a relatively high fraction. This species thus provides as powerful model to test theories related to sex chromosomes. Using this species, Carazo et al. (2016) recently provided the strongest evidence yet in favour of the unguarded X hypothesis. In their experiments, inbreeding reduced lifespan in both males and females, but the effect was more severe in females, effectively closing the gap in lifespan between the sexes at a high level of inbreeding. Alternative explanations to this pattern do however exist; the most prominent suggesting that inbreeding of the autosomes could have a relatively larger negative effect on females.

Here, we take advantage of genetic tools available for *D. melanogaster* to test the unguarded X hypothesis directly, by sampling a set of X chromosomes that we express in either an outbred or exclusively homozygous (effectively, completely inbred) state. We then compare their lifespan. Since the logic of the unguarded X extends to all traits under directional selection, we also test for an effect of X inbreeding on female egg-to-adult viability. In addition, we generate males and females that are outbred or exclusively homozygous for an autosome, and measure the impact of homozygosity on lifespan and egg-to-adult viability. Our results show a symmetrical negative effect on males and females, seen in both lifespan and viability, when the autosome is made homozygous. In contrast, we find no effect on either female lifespan or viability when the X chromosome is made homozygous. Our results thus provide no support in favour of the unguarded X hypothesis, and suggest that the effect of X-linked recessive deleterious alleles on sex differences in lifespan is small.

Material and Methods

Study Population

Flies used in our experiments came from the laboratory-adapted Dahomey population of *Drosophila melanogaster*. Dahomey is a wild-caught population collected from Dahomey (now Benin) in 1970 and kept since that point as a large outbred population, housed in population cages with overlapping generations under constant conditions (12:12h light-dark cycle, 25°C, 60% humidity, yeast and sugar-based food medium). We maintained these conditions during our experiments. While the population size of Dahomey is not strictly controlled, it is in the range of thousands of individuals per generation; this large size, overlapping generations and controlled conditions have contributed to it becoming a popular model for studies of lifespan and ageing.

Creation of Chromosome Homozygote Lines

The *D. melanogaster* genome consists of the sex chromosomes (X and Y), two major autosomes (A2 and A3) and the tiny A4 “dot” chromosome comprising <1% of the genome. Here, we generated lines that were exclusively homozygous for either the X chromosome or A2, hereafter referred to as X lines or A lines respectively. To create the lines, we relied on balancer chromosomes, which are genetic tools that suppress recombination with their homologue, and the fact that recombination does not occur in male *D. melanogaster*. This allowed us to sample focal X and A2 chromosomes at random from the Dahomey population, amplify them, and express them in either the heterozygous or exclusively homozygous state, in an otherwise randomly sampled Dahomey genetic background.

To create the X lines, we began by randomly collecting 28 males from the Dahomey population and mating them singly to groups of 3 virgin females carrying the compound X chromosome DX

(C[1]DX, y, f) in a variable Dahomey background. Sons from this cross inherit their X chromosome from the father and Y chromosome from the mother, meaning all male offspring from a given cross were isogenic for the focal X. For each line, these males were again mated to virgin DX-bearing females in larger groups to diversify the sample of Dahomey genetic variation elsewhere in the genome, and their sons were split into two groups. One group experienced a similar cross to the previous generation; in parallel, the other group was mated to virgin females heterozygous for an X chromosome balancer (FM7a, B^l , sc^8 , v^{Of} , w^a , y^{31d}) in a variable Dahomey background. Daughters of the latter cross inherit the focal X from their father, while half also receive the phenotypically marked balancer from their mother. To fix the focal X chromosomes, virgin balancer-bearing females from the latter cross were mated to sons from the former cross; female offspring not carrying the balancer were therefore homozygous for the focal X chromosome, while male offspring not carrying the balancer were hemizygous for it. One of the 28 lines was lost due to female sterility in the homozygous state. From the remaining lines, we randomly selected 20 that we maintained in 4 vials of 16 pairs, to preserve genetic variation elsewhere in the genome. For details, see Fig. S1A.

The creation of the A lines began by sampling 44 males at random from a replicate of the Dahomey population that was heterozygous for a marked A2 balancer (CyO, $Duox^{Cy}$, cn^2 , dpy^{lv} , pr^l), and mating them singly to groups of 5 virgin females heterozygous for the dominant A2 marker Kr^{Jf-1} in a variable Dahomey background. For each line, we collected sons from this cross that carried the Kr^{Jf-1} marker but not the balancer, and therefore also carried the focal A2. These sons were mated in groups to virgin females carrying the balancer heterozygously in a variable Dahomey background; because *D. melanogaster* males lack recombination, any offspring from this cross that do not express Kr^{Jf-1} carry the intact focal A2. We therefore collected sons and virgin daughters

that all carried the focal A2 opposite the balancer and mated them together, generating males and females that are fixed for the focal A2. Ten of the 44 lines were lost due to sterility/inviability in the homozygous state. From the remaining lines, we randomly selected 20 and maintained them in a similar manner to the X lines. For details, see Fig. S1B.

Lifespan Assay

We randomly selected 15 X and 15 A lines to assess lifespan. For each line, we measured the lifespan of 30 inbred and 30 outbred individuals of each sex, for a grand total of 3150 flies – note X line males are hemizygous and thus only measured once for each line. To avoid conflating maternal effects with the direct genetic effects of inbreeding, all experimental flies were produced by inbred females (Fig. 1). These females were collected as virgins from each X or A line after 2 generations of culture at a controlled density of 120 eggs per vial to standardize any environmental impact. For each line, 80 virgin females were mated to 80 males across 4 vials to generate inbred males and females, while another 80 virgin females were mated to 80 random Dahomey males in parallel to generate the matching outbred males and females (Fig. 1). After oviposition, the adults were discarded and the egg densities were again standardized to 120 per vial.

Ten days after oviposition, the experimental flies were collected under light CO₂ anesthesia. The 30 flies collected for each line, sex and inbreeding treatment were split into two vials of 15 each, and two days later we added 15 same-sex and 30 opposite-sex competitors to each vial; competitors were homozygous for a recessive phenotypic marker conferring dark body colouration (*ebony*, introgressed into the Dahomey background) and were ~11 days of age. For logistical reasons, competitor addition was taken as day 0 when measuring adult lifespan. In order to better standardize the demographic context when measuring lifespan, competitors were replaced with

new ~11 day old flies weekly under light CO₂ anesthesia. Flies were transferred to fresh vials 4 times a week (Mondays, Wednesdays, Thursdays and Saturdays), mortality was scored, and dead flies removed. Transfers were performed without anesthesia except during competitor replacement.

Egg-to-Adult Viability Assay

We measured egg-to-adult viability for the 15 X lines and 15 A lines used in the lifespan assay, plus an additional 5 lines of each type. To produce eggs for viability measurement, we used a similar crossing scheme to Fig. 1. For each line, 50 virgin females were combined with 25 males to produce inbred focal offspring, while another 50 virgin females were combined with 25 randomly selected Dahomey males to produce outbred focal offspring. Each group was allowed 5 days to mate in the presence of ad libitum live yeast to stimulate oviposition. Experimental eggs were collected in 4 blocks on successive days, generating four independent replicates; in each block, females were allowed to oviposit for 2-3 hours on a small petri dish of food medium. For each line, we collected 90 eggs per block and placed them together in a vial with 90 eggs from the ebony-marked competitor population laid in the same period. We scored egg-to-adult viability 12 days after oviposition by counting all focal and competitor adults of each sex that had successfully eclosed. Male and female egg-to-adult viability was thus estimated from the same sets of 90 eggs, under the assumption that an equivalent sex ratio at fertilization was produced in crosses generating inbred and outbred focal offspring. In this assay, hemizygous X line males were measured twice, as progeny of both the inbred and outbred crosses. We refer to these males as inbred and outbred here, but this only reflects their parentage.

Statistical Analysis

All statistical analyses were carried out using R (R Core Team, version R 3.3.2). To test for differences in variance between line means due to inbreeding, we used the Brown-Forsythe test for homogeneity of variances (a robust modification of Levene's test) using the package *car* and function *leveneTest* (Fox and Weisberg 2011). We used mixed-effect linear models in the package *lme4* to analyse the effects of sex and inbreeding on lifespan and viability (Bates et al. 2015). Tests for the significance of main effects and interactions were carried out using Type III Wald F-tests from the package *car* and function *Anova* (Fox and Weisberg 2011). To perform multiple comparisons of means, we used the package *lsmeans* and function *lsmeans* with pairwise contrasts and the Kenward-Roger approximation of degrees of freedom (Lenth 2016).

We assessed whether we had sampled a genetically variable set of X chromosomes and A2 chromosomes to found our lines with one-way, fixed-effect analysis of variance (ANOVA) using the base R function *aov*. In this analysis, performed both for lifespan and viability, a separate model was fit to each combination of sex and treatment (inbred/outbred), where line was the only factor. If line explained a significant portion of the trait variance, this was taken as evidence that the lines sampled were genetically variable.

Our crossing scheme, using balancer chromosomes to generate individuals that are homozygous for a given X chromosome or A2 chromosome, is efficient and based on well-established principles of *Drosophila* genetics. Nevertheless, we wished to confirm that we had successfully generated inbred lines, and to do so in a way that would be informative even if inbreeding did not result in depression of the life history traits we measured. We therefore tested for the increase in between-line variance expected between outbred and inbred genotypes (Robertson 1952). We performed this test separately for each chromosome, sex and trait combination, by estimating the variance

between line means in both the inbred and outbred state and comparing them using a Brown-Forsythe test for homogeneity of variances (package *car*).

We investigated the evidence for inbreeding depression of lifespan due to chromosome A2 inbreeding using a linear mixed-effects model (package *lme4*) with the following structure:

$$\text{lifespan} = \text{sex} + \text{treatment} + \text{sex} \times \text{treatment} + \text{line} + \text{line} \times \text{treatment} + \text{vial}(\text{sex} \times \text{treatment} \times \text{line})$$

where sex and treatment are fixed effects and the interaction between them models sex differences in the impact of inbreeding on lifespan. Line is a random effect, with a random slope estimated by the line x treatment interaction. Vial is a random effect that accounts for variance in lifespan between the two replicate vials for each combination of sex, treatment and line, and is explicitly nested within a combination.

Because males are hemizygous for the X chromosome, we were not able to fit a fully crossed model such as the one above to investigate inbreeding depression of lifespan in the X lines. We therefore fit a modified linear mixed-effects model (package *lme4*) with the structure below:

$$\text{lifespan} = \text{type} + \text{line} + \text{vial}(\text{type} \times \text{line})$$

where type is a fixed effect that contains joint information on sex and treatment, line is a random effect, and vial is a random effect that accounts for variance in lifespan between the two replicate vials for each combination of type and line. The effect of sex and inbreeding on lifespan was then decomposed using post-hoc contrasts. We also tested a random-slopes model containing the line x type interaction, but unfortunately the model did not converge so the term was omitted. Altering the use of line in this model does not adversely impact the fit, based on AIC (Akaike information criterion), likely due to the very small variance component attributable to line.

Egg-to-adult viability was modelled separately for X chromosomes and A2 chromosomes using linear mixed-effects models (package *lme4*) with the structure:

$$\text{viability} = \text{sex} + \text{treatment} + \text{sex} \times \text{treatment} + \text{line}$$

where the response was the number of adult individuals that successfully eclosed. Sex and treatment are fixed effects and their interaction tests for sex differences in the effect of inbreeding, while line is a random effect. As hemizygous X line males were measured twice, being produced by both the outbred and inbred crosses, we fit a fully crossed model to the X chromosome data to account for these two different parentages even though the males carried the same X in each case. We also tested models including the line x treatment interaction as a random slope, as well as block as a random factor accounting for variation between the days the experiment was replicated over. Including these terms did not improve the model's fit, based on AIC, so they were omitted. Viability data was also analysed using a generalized linear mixed-effect model with a binomial error distribution and logit link function (package *lme4*), with the response being the proportion of target adults to marked competitor adults eclosed. This approach produced qualitatively similar results.

Results

Genetic Variation

We detected significant genetic variation for lifespan among our X chromosome lines in males ($F = 1.909$, $df = 14$, $p = 0.024$), but not in either outbred ($F = 0.92$, $df = 14$, $p = 0.54$) or inbred ($F = 1.435$, $df = 14$, $p = 0.13$) females. In our set of autosomal lines, we detected significant genetic variation for lifespan in inbred females ($F = 7.034$, $df = 14$, $p < 0.001$) as well as in outbred ($F = 2.484$, $df = 14$, $p = 0.002$) and inbred males ($F = 6.866$, $df = 14$, $p < 0.001$), though not in outbred females ($F = 1.575$, $df = 14$, $p = 0.083$). For viability we found significant genetic variation for all sex and treatment combinations among both our X lines (outbred females: $F = 2.609$, $df = 19$, $p = 0.003$, inbred females: $F = 2.723$, $df = 19$, $p = 0.002$, outbred males: $F = 2.487$, $df = 19$, $p = 0.004$, inbred males: $F = 2.035$, $df = 19$, $p = 0.019$) and autosome lines (outbred females: $F = 2.502$, $df = 19$, $p = 0.004$, inbred females: $F = 2.445$, $df = 19$, $p = 0.005$, outbred males: $F = 4.245$, $df = 19$, $p < 0.001$, inbred males: $F = 4.285$, $df = 19$, $p < 0.001$). Note that in the case of X males, inbred and outbred here refer to the type of cross that generated the male offspring whose viability was measured; in both cases, males from a given line are hemizygous for the same X chromosome. These results show that we captured a genetically variable sample of X chromosomes and A2 chromosomes when founding our experimental lines.

Inbreeding

To confirm our lines had been successfully inbred, we tested for the increase in between-line variance expected under inbreeding (Robertson 1952). We found that between-line variance in lifespan increased due to inbreeding by at least a factor of 2.5 for each chromosome-sex combination. Using a Brown-Forsythe test this effect was however non-significant in all cases (all $p > 0.07$), probably due to a lack of power from having only 15 lines. Between-line variance in

viability also increased due to inbreeding in all chromosome-sex combinations, albeit not to the extent seen in lifespan (at least 1.07x for X chromosomes, at least 1.3x for A2 chromosomes). As with lifespan, no significant increases in variance were detected (all $p > 0.5$). Collectively, these results are consistent with both X and A2 chromosomes having been inbred successfully.

Lifespan

Lifespan of the X chromosome lines was significantly affected by type, the joint effect of sex and treatment ($F = 27.72$, $df = 2$, $p < 0.001$; Fig. 2A). This difference was attributable to males outliving both outbred and inbred females (respectively, $t = 7.01$, $df = 72.97$, $p < 0.001$ and $t = 5.68$, $df = 72.87$, $p < 0.001$). Crucially, inbreeding of the X chromosome did not affect lifespan in females ($t = 1.37$, $df = 72.90$, $p = 0.38$). In the autosome lines, there was a significant effect of both sex and treatment on lifespan (sex: $F = 8.475$, $df = 1$, $p = 0.011$, treatment: $F = 17.826$, $df = 1$, $p < 0.001$; Fig. 2B), with males again living longer than females, and inbreeding reducing lifespan. Notably, the interaction between sex and treatment was not significant ($F = 0.010$, $df = 1$, $p = 0.92$), indicating inbreeding the A2 chromosome reduced lifespan to a similar degree in each sex.

Viability

We did not find significant effects of sex, treatment, or their interaction on egg-to-adult viability in the X chromosome lines, (sex: $F = 0.522$, $df = 1$, $p = 0.47$, treatment: $F = 0.087$, $df = 1$, $p = 0.77$, sex x treatment: $F = 3.218$, $df = 1$, $p = 0.074$; Fig. 2C). This means that, as was the case for lifespan, inbreeding the X chromosome does not result in a reduction in female viability. In the autosome lines, we found that egg-to-adult viability did not differ between the sexes but there was a significant effect of inbreeding (sex: $F = 0.493$, $df = 1$, $p = 0.34$, treatment: $F = 7.49$, $df = 1$, $p = 0.007$; Fig. 2D), with inbred individuals suffering reduced viability. There was no significant

interaction between sex and viability, indicating inbreeding the A2 chromosome reduces the viability of males and females to a similar degree ($F = 0.271$, $df = 1$, $p = 0.60$).

Discussion

Here we tested if X-linked recessive deleterious mutations play an important role in shaping the relative lifespan of males and females, as postulated by the unguarded X hypothesis. We conducted our experiments using a *D. melanogaster* laboratory population cultured at a large effective population size with overlapping generations, which suggests that recessive mutations with negative effects on lifespan should segregate at mutation-selection balance. After producing flies that are exclusively homozygous for the X chromosome, we do not detect a resulting decline in either female lifespan or egg-to-adult viability. In contrast, inbreeding of an autosome has a substantial negative effect on lifespan as well as egg-to-adult viability in both sexes. Collectively these results suggest X-linked recessive deleterious alleles should only have a small negative influence on male lifespan in this population, and that the unguarded X hypothesis probably cannot explain general sex differences in lifespan.

In both our X chromosome and autosome assays, we find that males outlive females. This was surprising, since in previous studies of this population we and others (e.g. Megwere et al. 1994; Lehtovaara et al. 2013; Carazo et al. 2016; Griffin et al. 2016; Duxbury et al. 2017) have found that females outlive males. The degree of exposure to males does, however, have a large impact on survival of *D. melanogaster* females (Partridge et al. 1987; Friberg 2005; Lethovaara et al. 2013; Zajitschek et al. 2013), and during the experiments we replaced competitor flies that focal individuals were housed with on a weekly basis. This is not standard procedure in lifespan experiments but seems like the most relevant protocol, since flies in stable populations with overlapping generations experience a constant demographic structure throughout their lives. The shorter lifespan of females is therefore most likely explained by the constant exposure to young males. Importantly, the fact that males outlived females has no influence on our ability to evaluate

the unguarded X hypothesis as a general mechanism shortening male lifespan, since the test is exclusively based on comparing the lifespan of X inbred and outbred females.

Our finding that inbreeding the X chromosome has no effect on egg-to-adult viability is consistent with an earlier contribution. Eanes et al. (1985) conducted a large study on two independently wild-caught sets of 50 and 90 X chromosomes, respectively, for which they measured egg-to-adult viability in their outbred and inbred state. No evidence for a negative effect of inbreeding on viability was found in either set. Similarly to our study, their inbred flies were generated through the use of balancer chromosomes. This method guarantees that no genetic variants other than recessive lethal and some semi-lethal mutations are purged. Our studies should therefore not have underestimated the effect of recessive deleterious mutations, as may happen when their effect is assessed through inbred lines generated via multiple generations of full-sib crosses since this method is associated with more purging (Hedrick 1994). While consistent with other empirical data, our results stand in contrast to population genetic models, which predict that sex-specific mortality could be non-trivially affected at mutation-selection balance when X-linked deleterious mutations are completely recessive (Pipoly et al. 2015). Current data do, however, suggest that X-linked deleterious mutations are only partly recessive on average ($h \sim 0.3$) (Eanes et al. 1985; Mallet et al. 2011). This means selection can act against deleterious mutations in the homogametic sex as well, keeping their frequency and relative effect on the hemizygous sex lower. Collectively, our results and those of others thus suggest that X-linked recessive alleles with negative effects on egg-to-adult viability are rare and segregate at a low frequency.

Given that selection and/or mutation keeps the frequency of X-linked recessive alleles with deleterious effects on egg-to-adult viability low, it is not surprising that female lifespan also shows

no effect of X inbreeding in our study. This finding does, however, differ from the results obtained by Carazo et al. (2016), who found that inbreeding of the whole genome (generated by 10 generations of full-sib crosses) substantially shortened female compared to male lifespan. The authors attributed this asymmetry in inbreeding depression to the X chromosome, since the autosomes were inbred to the same extent in both sexes and the X only in females. Inbreeding of the autosomes does not, however, necessarily result in equal inbreeding depression in both sexes. Studies have frequently shown that inbreeding depression differs between males and females (reviewed in Ebel and Phillips 2016), with no consistent direction with respect to the homogametic sex. In the population studied by both us and Carazo et al. the genetic architecture of lifespan has previously been shown to differ substantially between the sexes, across the whole genome (Lehtovaara et al. 2013) and for the autosomes exclusively (Griffin et al. 2016). Sex-specific effects on lifespan by autosomal inbreeding would therefore not be surprising. That said, we find no support for this hypothesis here, since inbreeding depression for lifespan measured through an autosome was almost identical for males and females in our experiments. Our autosomal results may, however, not be entirely conclusive since we only studied one of the two major autosomes, leaving the possibility that sex-specific effects of inbreeding are limited to the other major autosome.

Another potential cause of the divergence between our results and those of Carazo et al. (2016) stems from differences in the experimental design coupled with the context-dependent nature of inbreeding effects (Fox and Reed 2010; Yun and Agrawal 2014). While focal males and females in our experiments were kept in moderate-sized, mixed-sex groups and allowed to mate, those of Carazo et al. were kept as virgins in smaller same-sex groups, providing fundamentally different environmental contexts over life. Likely due to this, outbred females in the two studies had different

average lifespans (~50 days in ours, ~71 in Carazo et al.), and differences between our results and theirs could be produced if expression of recessive deleterious alleles is confined primarily to very late life. Alternatively, the lack of an effect in our study could potentially occur if recessive deleterious mutations have more pronounced effects in the less stressful environment studied by Carazo et al. (but see Fox and Reed 2010). It could also occur because males court high quality females more vigorously than low quality females (Long et al. 2009), diminishing any inherent differences in lifespan between inbred and outbred genotypes if the latter are higher quality. The fact that we see no effect of X inbreeding on egg-to-adult viability, and a clear effect of autosomal inbreeding on both egg-to-adult viability and lifespan, does however speak against these explanations.

In any case, if the results obtained by Carazo et al. were caused by recessive X-linked deleterious alleles, their effect must have been substantial. In their experiments, male lifespan dropped from ~62 to ~58 days (~6.5%) and female lifespan from ~71 to ~57 days (~20%) due to inbreeding. Assuming the autosomal effect is similar in males and females, female lifespan should have dropped ~5 days due to autosomal inbreeding alone, leaving inbreeding of the X to account for the remaining ~9 days. A larger effect of the X is inconsistent with the fact that the X is only one quarter of the size of the autosomal genome, and that it has probably been purged of deleterious alleles to a larger extent because of their unconditional exposure in males. These numbers are estimated from means associated with uncertainties, but suggest that the larger drop in female lifespan found by Carazo et al. was at least not entirely explained by expression of recessive deleterious alleles on the X, unless X and autosomal inbreeding had a strong negative non-additive effect on female lifespan.

While the unguarded X predicts a reduction in female lifespan due to inbreeding, this prediction is not unique to the unguarded X. Other potential causes of the same pattern are overdominance (Wilton and Sved 1979; Connallon and Knowles 2006) and recessive alleles with female-limited deleterious effects. Since overdominance on the X (or Z) cannot occur in the heterogametic sex, this essentially represents a fourth hypothesis to explain why the homogametic sex is generally more long-lived. Overdominance is, however, believed to have a very small effect on inbreeding depression relative to recessive deleterious alleles (Charlesworth and Willis 2009), so this possibility probably can be dismissed (but see Connallon and Knowles 2006). If reduction in female performance due to X inbreeding is observed, recessive alleles with female-specific deleterious effects are a more plausible cause. The X chromosome is enriched in genes with female versus male-biased expression in *D. melanogaster* (Ranz et al. 2003) and mammals (Khil et al. 2004; Reinius et al. 2012), and in flies the estimated X-linked intersexual genetic correlation based on standing genetic variation is low for both fitness (Mallet et al. 2011) and lifespan (Griffin et al. 2016). This suggests that X-linked inbreeding depression could result exclusively from recessive alleles with deleterious effects limited to females. Truly convincing evidence for the unguarded X may therefore require demonstrating that the heterogametic sex lives longer when supplemented with an extra “guarding” X (or Z) chromosome. This is technically possible in *D. melanogaster*, but interpretation of the results would be complicated by the fact that males with an XX (or XXY) karyotype are not completely masculinized (Casper and Van Doren 2006; Camara et al. 2008).

Given that X-linked recessive alleles with deleterious effects limited to the homogametic sex could cause a reduction of lifespan in the homogametic sex following X inbreeding, a strong test of the unguarded X hypothesis using X inbreeding should survey other traits in addition to lifespan. These should be traits for which the sexes share a common genetic architecture, and which are under

directional selection in both sexes. In *D. melanogaster*, egg-to-adult viability is such a trait (Chippindale et al. 1999), and here we tested for an effect of X inbreeding on this trait alongside lifespan. Neither of these traits showed any evidence of a decline in X inbred females, despite the fact that the X comprises as much as 20% of the genome in this species. From these results, we conclude that the segregating X-linked recessive deleterious alleles fundamental to the unguarded X hypothesis explain, at best, a small fraction of sex differences in lifespan. This conclusion rests on experiments using a single laboratory-adapted population. To test if it holds beyond our study population, further research should focus on other populations, ideally ones more recently sampled from the wild. Using laboratory-adapted populations comes with the advantage that lifespan can be measured in a relevant environment, but recessive deleterious alleles might be maintained at artificially low frequencies because of adaptation to a static environment. Our study at least partly overcomes this potential problem, since it contrasts the relative impact of X-linked to autosomal segregating recessive deleterious alleles, and may therefore be generally applicable to other populations.

Literature Cited

- Adler, M. I., and R. Bonduriansky. 2014. Sexual conflict, life span, and aging. Cold Spring Harb. Perspect. Biol. 6:a017566.
- Archer, C. R., F. Zajitschek, S. K. Sakaluk, N. J. Royle, and J. Hunt. 2012. Sexual selection affects the evolution of lifespan and ageing in the decorated cricket *Gryllodes sigillatus*. Evolution 66:620-634.
- Austad, S. N., and K. E. Fischer. 2016. Sex differences in lifespan. Cell Metab. 23:1022-1033.
- Bates, D., M. Mächler, B. M. Bolker, and S. C. Walker. 2015. Fitting linear mixed-effects models using lme4. J. Stat. Soft. 67:1-48.
- Bilde, T., A. A. Maklakov, L. de la Guardia, K. Meisner, and U. Friberg. 2009. Sex differences in the genetic architecture of a life history trait: extreme inbreeding increases male lifespan in a seed beetle. BMC Evol. Biol. 9:33.
- Bonduriansky, R., A. Maklakov, F. Zajitschek, and R. Brooks. 2008. Sexual selection, sexual conflict and the evolution of ageing and life span. Funct. Ecol. 22:443-453.
- Camara, N., C. Whitworth, and M. Van Doren. 2008. The creation of sexual dimorphism in the *Drosophila* soma. Curr. Top. Dev. Biol. 83:65-107.
- Camus, F., D. J. Clancy, and D. K. Dowling. 2012. Mitochondria, maternal inheritance, and male aging. Curr. Biol. 22:1717-1721.
- Carazo, P., J. Green, I. Sepil, T. Pizzari, and S. Wigby. 2016. Inbreeding removes sex differences in lifespan in a population of *Drosophila melanogaster*. Biol Lett. 12: 20160337.
- Casper, A., and M. Van Doren. 2006. The control of sexual identity in the *Drosophila* germline. Development 133:2783-2791.

- Charlesworth, D., and J. H. Willis. 2009. The genetics of inbreeding depression. *Nat. Rev. Genet.* 10:783-796.
- Chippindale, A. K., J. R. Gibson, and W. R. Rice. 2001. Negative genetic correlation for adult fitness between sexes reveals ontogenetic conflict in *Drosophila*. *Proc. Natl. Acad. Sci. USA* 98:1671-1675.
- Comfort, A. 1979. *The biology of senescence*. Elsevier, New York, USA.
- Connallon T., and L. L. Knowles. 2016. Evidence for overdominant selection maintaining X-linked fitness variation in *Drosophila melanogaster*. *Evolution* 60:1445-1453.
- Duxbury, E. M. L., W. G. Rostant, and T. Chapman. 2017. Manipulation of feeding regime alters sexual dimorphism for lifespan and reduces sexual conflict in *Drosophila melanogaster*. *Proc. Roy. Soc. B Biol. Sci.* 284:20170391.
- Eanes, W. F., J. Hey, and D. Houle. 1985. Homozygous and hemizygous viability variation on the X chromosome of *Drosophila melanogaster*. *Genetics* 111:831-844.
- Ebel, E. R., and P. C. Phillips. 2016. Intrinsic differences between male and females determine sex-specific consequences of inbreeding. *BMC Evol. Ecol.* 16:36.
- Frank, S. A., and L. D. Hurst. 1996. Mitochondria and male disease. *Nature* 383:224.
- Finch, C. E. 1990. *Longevity, senescence, and the genome*. University of Chicago Press, Chicago, USA.
- Flatt, T. 2011. Survival costs of reproduction in *Drosophila*. *Exp. Gerontol.* 46:369-375.
- Friberg, U. 2005. Genetic variation in male and female reproductive characters associated with sexual conflict in *Drosophila melanogaster*. *Behav. Genet.* 35:455-462.
- Fox, C. W., and D. H. Reed. 2010. Inbreeding depression increases with environmental stress: an experimental study and meta-analysis. *Evolution* 65:246-258.

- Fox, C. W., K. L. Scheibly, W. G. Wallin, L. J. Hitchcock, R. C. Stillwell, and B. P. Smith. 2006. The genetic architecture of life span and mortality rates: gender and species differences in inbreeding load of two seed-feeding beetles. *Genetics* 174:763-773.
- Fox, J., and S. Weisberg. 2011. *An R companion to applied regression*. SAGE, Thousand Oaks, CA.
- Gemmell, N. J., V. J. Metcalf, and F. W. Allendorf. 2004. Mother's curse: the effect of mtDNA on individual fitness and population viability. *Trends Ecol. Evol.* 19:238–244.
- Graves, B. M. 2007. Sexual selection effects on the evolution of senescence. *Evol. Ecol.* 21:663-668.
- Griffin, R. M., H. Schielzeth, and U. Friberg. 2016. Autosomal and X-linked additive genetic variation for lifespan and aging: comparisons within and between the sexes in *Drosophila melanogaster*. *G3* 6:3903-3911.
- Hedrick, P. W. 1994. Purging inbreeding depression and the probability of extinction: full-sib mating. *Heredity* 73:363-372.
- Innocenti, P., E. Morrow, and D. K. Dowling. 2011. Experimental evidence supports a sex-specific selective sieve in mitochondrial genome evolution. *Science* 332:845-848.
- Khil, P. P., N. A. Smirnova, P. J. Romanienko, and R. D. Camerini-Otero. 2004. The mouse X chromosome is enriched for sex-biased genes not subject to selection by meiotic sex chromosome inactivation. *Nat. Genet.* 36:642–646.
- Lehtovaara, A., H. Schielzeth, I. Flis, and U. Friberg. 2013. Heritability of lifespan is largely sex-limited in fruit flies. *Am. Nat.* 182:653-665.
- Lenth, R. V. 2016. Least-squares means: the R package lsmeans. *J. Stat. Soft.* 69:1-33.
- Liker, A. and T. Szekely. 2005. Mortality costs of sexual selection and parental care in natural populations of birds. *Evolution* 59:890–897.

- Long, T. A. F., A. Pischedda, A. D. Stewart, and W. .R. Rice. 2009. A cost of sexual attractiveness to high-fitness females. PLoS Biology 7 (12), e1000254.
- Maklakov, A. A., V. Lummaa. 2013 Evolution of sex differences in lifespan and aging: causes and constraints. Bioessays 35:717–724.
- Mallet, M. A., J. M. Bouchard, C. M. Kimber, and A. K. Chippindale. 2011. Experimental mutation-accumulation on the X chromosome of *Drosophila melanogaster* reveals stronger selection on males than females. BMC Evol. Biol. 11:156.
- Medawar, P. B. 1952. An unsolved problem in biology. H.K. Lewis, London.
- Magwere, T., T. Chapman, and L. Partridge. 2004. Sex differences in the effect of dietary restriction on life span and mortality rates in female and male *Drosophila melanogaster*. J. Gerontol. A Biol. Sci. Med. Sci. 59:3-9.
- Partridge, L., A. Green, and K. Fowler. 1987. Effects of egg production and of exposure to males on female survival in *Drosophila melanogaster*. J. Insect Physiol. 33:745-749.
- Pipoly, I., V. Bokony, M. Kirkpatrick, P. F. Donald, T. Szekely, and A. Liker. 2015. The genetic sex-determination system predicts adult sex ratios in tetrapods. Nature 527:91–94.
- Promislow, D. 2003. Mate choice, sexual conflict, and evolution of senescence. Behav. Genet. 33: 191-201.
- Promislow, D. E. L. 1992. Costs of sexual selection in natural populations of mammals. Proc. Roy. Soc. B Biol. Sci. 247:203-210.
- Promislow, D. E. L., R. Montgomerie, and T.E. Martin. 1992. Mortality costs of sexual dimorphism in birds. Proc. Roy. Soc. B Biol. Sci. 250:143-150.
- R Core Team. 2016. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. <http://www.R-project.org>.

- Ranz, J. M., C. I. Castillo-Davis, C. D. Meiklejohn, and D. L. Hartl. 2003. Sex-dependent gene expression and evolution of the *Drosophila* transcriptome. *Science* 300:1742-1745.
- Reinius, B., M. M. Johansson, K. J. Radomska, E. H. Morrow, G. K. Pandey, C. Kanduri, R. Sandberg, R. W. Williams, and E. Jazin. 2012. Abundance of female-biased and paucity of male-biased somatically expressed genes on the mouse X-chromosome. *BMC Genomics* 13:607.
- Robertson, A. 1952. The effect of inbreeding on the variation due to recessive genes. *Genetics* 37: 189-207.
- Rolff, J. 2002. Bateman's principle and immunity. *Proc. Roy. Soc. B Biol. Sci.* 268: 867-872.
- Svensson, E., and B. C. Sheldon. 1998. The social context of life history evolution. *Oikos* 83:466–477.
- Trivers, R. 1985. *Social evolution*. Benjamin/Cummings Publishing, Menlo Park, CA.
- Willton, A. N., and J. A. Sved. 1979. X-chromosome heterosis in *Drosophila melanogaster*. *Genet. Res. Int.* 34:303-315.
- Williams, G. C. 1957. Pleiotropy, natural selection, and the evolution of senescence. *Evolution* 11: 398–411.
- Zajitschek, F., S. R. K. Zajitschek, U. Friberg, and A. A. Maklakov. 2013. Interactive effects of sex, social environment, dietary restriction and methionine on survival and reproduction in fruit flies. *AGE* 35: 1193-1204.
- Yun, L., and A. F. Agrawal. 2014. Variation in the strength of inbreeding depression across environments: effects of stress and density dependence. *Evolution* 68:3599–3606.

Figure Legends

Figure 1: Crossing design to produce matched inbred and outbred genotypes for (A) the X chromosome and (B) the autosome A2. Each larger rectangle depicts an individual/genotype with the sex chromosomes (Y as the letter Y and X as a smaller rectangle) and the two major autosomes (A2 and A3) displayed within; the A4 “dot” chromosome is omitted from the figure for clarity. Blue and green chromosomes depict a focal X chromosome and A2 chromosome, respectively, while white rectangles depict chromosomes with copies sampled from the base Dahomey population that vary randomly between individuals. Focal inbred and outbred male and female genotypes (second row in A and B) were both generated using the inbred female genotype as dam, to standardize maternal effects between inbred and outbred flies for each matched pair. These females were mated to either random Dahomey males to generate outbred focal flies, or to males carrying the cloned chromosome copy (first row in A and B) to generate inbred focal flies. See Figure S1 for generation of parental flies used in these crosses.

Figure 2: Mean adult lifespan measured in days is shown for (A) the X lines and (B) the A lines. As the X is hemizygous in males, only a single measurement of X line longevity in males was made. This is rendered as outbred as it represents expression in outbreeding populations. Egg-to-adult viability is shown in (C) for X lines and (D) for A lines, measured as the number of eclosed adults of each sex from a fixed number of eggs. In this experiment, viability of hemizygous X line males was measured twice (called inbred and outbred in the figure to clarify from which cross they were generated). In all cases, the *x*-axes show whether the focal chromosome was heterozygous (outbred) or homozygous (inbred). Points represent the grand mean of individual line means, while error bars represent the standard error of the grand mean. Closed points show female trait values while open points show male values.

Generating matched inbred and outbred X genotypes

Generating matched inbred and outbred A genotypes

A **B** **C** **D**

● Female ○ Male

Figure S1A.

Crosses used to create lines homozygous for the X chromosome

- A single Dahomey male crossed to 3 virgin DX-Dahomey females, to capture a single X chromosome copy.
- One vial with 5 males crossed to 15 virgin DX-Dahomey females, made to amplify focal X and diversify the genetic background.
- Left: One vial with 10 males crossed to 15 virgin DX-Dahomey females, to further diversify genetic background. Right: Two vials with 5 males and 10 virgin FM-Dahomey females, to balance focal X copy in females.
- One vial with 16 males and 16 females with focal X balanced over FM, to produce line homozygous for focal copy of X.
- 16 males and 16 females collected for the first generation. Line then maintained at 4 vials with 16 pairs in each, to maintain the focal X in a diverse genetic background.

Of the 28 X chromosomes we attempted to clonally amplify and make X inbred lines from, one was lost due to female sterility in the homozygous state. 20 of the inbred lines were randomly chosen for the assay of egg-to-adult viability, and 15 of these were randomly chosen for the lifespan assay. To generate focal experimental males and females, inbred females from each line were subsequently crossed either to males carrying the same copy of the X chromosome (essentially cross E) or to random Dahomey males, where the first cross generated inbred males and females and the latter outbred males and females. See Figure 1 for these crosses.

Figure S1B.

Crosses used to create lines homozygous for chromosome A2

- Single Dahomey male carrying a copy of CyO crossed to 5 virgin *Kr^{lf-1}*-Dahomey females, to amplify focal A2, place it over *Kr^{lf-1}* in sons, and diversify the genetic background.
- Two vials with 5 males with the focal A2 copy balanced over *Kr^{lf-1}*, crossed to 10 virgin CyO-Dahomey females, to balance focal A2 over CyO in both sexes and diversify genetic background.
- One vial with 16 males and 16 virgin females with focal A2 balanced over CyO, to produce line homozygous for focal copy of A2.
- 16 males and 16 females collected for the first generation. Line then maintained at 4 vials with 16 pairs in each, to maintain the focal A2 in a diverse genetic background.

Of the 44 A2 chromosomes we attempted to clonally amplify and make A inbred lines from, ten were lost due to sterility or inviability in the homozygous state. 20 of the inbred lines were randomly chosen for the assay of egg-to-adult viability, and 15 of these were randomly chosen for the lifespan assay. To generate focal experimental males and females, inbred females from each line were subsequently crossed either to males carrying the same inbred copy of the A2 chromosome (essentially cross D) or to random Dahomey males, where the first cross generated inbred males and females and the latter outbred males and females. See Figure 1 for these crosses.

The Y chromosome associated with the balancer in cross A was not of Dahomey origin. This Y chromosome was inherited through all crosses to the inbred line. Therefore inbred focal males, generated from crossing inbred males and females, had a different Y chromosome than outbred males generated from crossing inbred females to random Dahomey males.

Figure S1A.

Crosses used to create lines homozygous for the X chromosome

- A single Dahomey male crossed to 3 virgin DX-Dahomey females, to capture a single X chromosome copy.
- One vial with 5 males crossed to 15 virgin DX-Dahomey females, made to amplify focal X and diversify the genetic background.
- Left: One vial with 10 males crossed to 15 virgin DX-Dahomey females, to further diversify genetic background. Right: Two vials with 5 males and 10 virgin FM-Dahomey females, to balance focal X copy in females.
- One vial with 16 males and 16 females with focal X balanced over FM, to produce line homozygous for focal copy of X.
- 16 males and 16 females collected for the first generation. Line then maintained at 4 vials with 16 pairs in each, to maintain the focal X in a diverse genetic background.

Of the 28 X chromosomes we attempted to clonally amplify and make X inbred lines from, one was lost due to female sterility in the homozygous state. 20 of the inbred lines were randomly chosen for the assay of egg-to-adult viability, and 15 of these were randomly chosen for the lifespan assay. To generate focal experimental males and females, inbred females from each line were subsequently crossed either to males carrying the same copy of the X chromosome (essentially cross E) or to random Dahomey males, where the first cross generated inbred males and females and the latter outbred males and females. See Figure 1 for these crosses.

Figure S1B.

Crosses used to create lines homozygous for chromosome A2

- Single Dahomey male carrying a copy of CyO crossed to 5 virgin *Kr^{lf-1}*-Dahomey females, to amplify focal A2, place it over *Kr^{lf-1}* in sons, and diversify the genetic background.
- Two vials with 5 males with the focal A2 copy balanced over *Kr^{lf-1}*, crossed to 10 virgin CyO-Dahomey females, to balance focal A2 over CyO in both sexes and diversify genetic background.
- One vial with 16 males and 16 virgin females with focal A2 balanced over CyO, to produce line homozygous for focal copy of A2.
- 16 males and 16 females collected for the first generation. Line then maintained at 4 vials with 16 pairs in each, to maintain the focal A2 in a diverse genetic background.

Of the 44 A2 chromosomes we attempted to clonally amplify and make A inbred lines from, ten were lost due to sterility or inviability in the homozygous state. 20 of the inbred lines were randomly chosen for the assay of egg-to-adult viability, and 15 of these were randomly chosen for the lifespan assay. To generate focal experimental males and females, inbred females from each line were subsequently crossed either to males carrying the same inbred copy of the A2 chromosome (essentially cross D) or to random Dahomey males, where the first cross generated inbred males and females and the latter outbred males and females. See Figure 1 for these crosses.

The Y chromosome associated with the balancer in cross A was not of Dahomey origin. This Y chromosome was inherited through all crosses to the inbred line. Therefore inbred focal males, generated from crossing inbred males and females, had a different Y chromosome than outbred males generated from crossing inbred females to random Dahomey males.