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Cite this article: Flanagan BA, Li N, Edmands S. 2021 Mitonuclear interactions alter sex-specific longevity in a species without sex chromosomes. *Proc. R. Soc. B* **288**: 20211813. https://doi.org/10.1098/rspb.2021.1813

Received: 13 August 2021
Accepted: 11 October 2021

Subject Category:
Evolution

Subject Areas:
evolution, genetics

Keywords:
Tigriopus californicus, ageing, hybridization, DNA damage, sex ratio

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Mitonuclear interactions alter sex-specific longevity in a species without sex chromosomes

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Impaired mitochondrial function can lead to senescence and the ageing phenotype. Theory predicts degenerative ageing phenotypes and mitochondrial pathologies may occur more frequently in males due to the matrilineal inheritance pattern of mitochondrial DNA observed in most eukaryotes. Here, we estimated the sex-specific longevity for parental and reciprocal F1 hybrid crosses for inbred lines derived from two allopatric *Tigriopus californicus* populations with over 20% mitochondrial DNA divergence. *T. californicus* lacks sex chromosomes allowing for more direct testing of mitochondrial function in sex-specific ageing. To better understand the ageing mechanism, we estimated two age-related phenotypes (mtDNA content and 8-hydroxy-20-deoxyguanosine (8-OH-dG) DNA damage) at two time points in the lifespan. Sex differences in lifespan depended on the mitochondrial and nuclear backgrounds, including differences between reciprocal F1 crosses which have different mitochondrial haplotypes on a 50 : 50 nuclear background, with nuclear contributions coming from alternative parents. Young females showed the highest mtDNA content which decreased with age, while DNA damage in males increased with age and exceed that of females 56 days after hatching. The adult sex ratio was male-biased and was attributed to complex mitonuclear interactions. Results thus demonstrate that sex differences in ageing depend on mitonuclear interactions in the absence of sex chromosomes.

1. Introduction

Species with separate sexes typically exhibit ageing dimorphism where one sex experiences greater mortality than the other [1,2]. Many explanations, both proximate and ultimate, have been proffered for these sex differences. In species with heteromorphic sex chromosomes, the homogametic sex typically lives longer [3] suggesting the expression of unguarded deleterious variants in the heterogametic sex may drive lifespan reduction. But many taxa have separate sexes without sex chromosomes [4], and such taxa allow characterization of additional factors underlying sex differences in lifespan and ageing including sex-specific trade-offs between mortality and reproduction (e.g. [5]).

Mitochondrial function has been associated with ageing [6,7] and may explain sex differences in lifespan [8] and contribute to the development of age-related disease [9]. The mitochondrial genome (mtDNA) is almost exclusively maternally inherited in bilaterians. Due to the matrilineal inheritance patterns, mtDNA variants could arise in a population that negatively impact male fitness, yet those variants may not be purged from the population through natural selection because males do not transmit their mtDNA to the next generation [10,11]. Natural selection can operate in females; therefore, male harming mtDNA mutations may accumulate if they fail to decrease female fitness, referred to as the Mother's Curse [11]. Although under thermally stressful conditions, functional mitochondrial variation can result in sexually concordant selection [12]. The most rigorous tests of the Mother's Curse hypothesis have

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been conducted in *Drosophila*, where some studies find support for the hypothesis (e.g. [13–18]) while others do not (e.g. [19,20]). Therefore, the Mother's Curse genetic conflict is highly variable across eukaryotes and may be difficult to detect due to the nuclear suppression of sexually antagonistic mitochondrial variants [21].

Mitochondria are involved in metabolism for both sexes, yet the metabolic demands for males and females often differ. Oxidative phosphorylation (OXPHOS) produces usable energy and relies upon the functional coordination of over 1000 nuclear and 13 mitochondrial protein-encoded genes [22,23]. OXPHOS complexes have subunits encoded by both nuclear and mitochondrial genes. The nuclear products are synthesized in the cytosol and then imported to the mitochondria to form functional proteins [24]. When genetic incompatibilities are exposed either in late-generation interpopulation hybrids [25] or known mitonuclear combinations [26], animals harbouring incompatibilities show phenotypes associated with altered OXPHOS efficiencies including increased oxidative damage to DNA [25] and elevated levels of hydrogen peroxide [26].

Males and females may show different levels of DNA damage because reactive oxygen species (ROS) produced by leaky OXPHOS enzymes depend on metabolic demand which may differ between the sexes. Even though ROS are important in cellular signalling [27], if production exceeds the equilibrium capacity of the reducing chemicals and enzymes, the organism experiences oxidative stress. Oxidative stress can damage OXPHOS adjacent cellular macromolecules including the mtDNA [28]. Oxidative damage to macromolecules lies at the centre of one of the most highly tested ageing theories, the free radical theory of ageing [29,30]. Multiple critiques of the free radical theory of ageing exist (e.g. [31–33]), including evidence that ROS produced by a mitochondrion can induce hormesis (mitohormesis) where low levels of cellular stress induce a physiological change which has a positive relationship to stress tolerance later in life and can result in an increased lifespan [34].

Mitochondrial copy number (mtDNA content) often differs between sexes [35] and may shed light on sex differences in lifespan. mtDNA copy number has been proposed as a biomarker that negatively correlates with age [36,37] and positively associates with physiological robustness [36]. Mitochondrial numbers are dynamic and depend on biogenesis and mitophagy, both of which are influenced by ROS [38,39]. mtDNA content has been found to both increase and decrease with increased ROS levels. For example, mitochondrial malfunction in *Drosophila* has been shown to increase both ROS levels and mtDNA content [40] while in a human diseased state associated with decreased mtDNA content, ROS levels were higher than under healthy conditions [41]. Therefore, the relationship between mtDNA content and ROS levels remains unclear [42].

Here, we use the harpacticoid copepod *Tigriopus californicus* to explore the mitochondrial effects of sex-specific ageing. *T. californicus* occupies supralittoral zone of rocky shores from northern Mexico through southern Alaska [43] where populations show high-genetic differentiation even when separated by geographic short distances [43–45], and the mitochondrial sequence divergence between populations can exceed 20% [43,46]. Sex determination in *T. californicus* does not rely on sex chromosomes, and instead, sex is determined polygenically where multiple loci throughout the genome

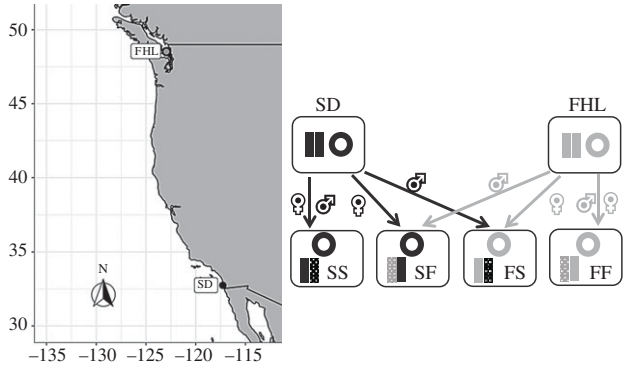

Figure 1. Geographic locations of populations used in this study—SD (San Diego, CA), FHL (Friday Harbor Labs, WA)—and reciprocal hybrid cross design. Solid bars indicate nuclear alleles contributed by the mother and the dotted bars indicate nuclear alleles contributed by the father.

contribute to sex [47–49]. Like other bilaterians, *T. californicus* displays maternal mtDNA inheritance [49].

Even though *T. californicus* lacks sex chromosomes, sex differences are apparent in this species. Females are typically more tolerant than males when exposed to a multitude of abiotic stressors including high salinity, low salinity and high temperature [50]. Foley *et al*. [50] reported no difference in sex-specific longevity except in one replicate at high temperature where median longevity was slightly higher in females. Also, when challenged by both chronic [51] and acute [52] exposure to exogenous oxidative stress, males and females had divergent transcriptomic responses, suggesting that longevity and age-related phenotypes may differ between the sexes.

Characterizing sex-specific ageing in a species without sex chromosomes provides a simpler system to probe mitochondrial effects. Sex bias in mitochondrial effects can be confounded with the effects of sex chromosomes, which can have sex-specific effects due to both asymmetric inheritance and incomplete dosage compensation [53,54]. Further, the movement of genes on or off sex chromosomes can both cause and resolve sexual conflicts [53,55]. Here, we build *T. californicus* as an alternative invertebrate ageing model first by robustly estimating sex-specific lifespan using reciprocal F1 hybrid crosses from two populations with 20.6% mtDNA divergence [46]. By using reciprocal hybrid crosses that have a 50 : 50 nuclear background with alternative mitochondrial haplotypes and nuclear contributions coming from alternative parents, we can more directly test mitochondrial effects. Then, because mitochondrial malfunction can lead to macromolecule damage and senescence [6,7], we estimate two age-related phenotypes (8-hydroxy-2′-deoxyguanosine (8-OH-dG) DNA damage and mtDNA content) longitudinally for two age classes. These results will have implications for human health and will further our understanding of how mitochondria contribute to sex-specific ageing.

2. Material and methods

(a) Population sampling and culture maintenance

Copepods were collected from supralittoral pools at San Diego, CA, USA (SD; 32.74 N 117.25 W) and Friday Harbor Labs, WA, USA (FHL; 48.55 N 123 W; figure 1), and we established inbred lines under full-sib mating for at least 10 generations before the

experiment began. All animals were maintained at 20°C in the same incubator with a 12 : 12 light : dark cycle. During the experiment, animals were fed ground Spirulina (Nutraceutical Science Institute, USA) and ground Tetramin fish food (Tetra Holding Inc., USA) with each at a concentration of 0.1 g per litre of seawater. Seawater used in this experiment was collected from the USC Wrigley Marine Science Center (Catalina Island, CA, USA) and was triple filtered using a 37 µm filter.

(b) Experimental crosses, longevity and fertility estimates

Using inbred lines from SD and FHL, we generated parental (FF and SS) and reciprocal hybrid crosses (FS and SF) (figure 1) where the first letter represents the female parental line while the second represents the male parental line. *T. californicus* adult males perform a mate-guarding behaviour where they clasp virgin females until the females become reproductively receptive [56]. Importantly, unmated females that reach sexual maturity can still mate and produce viable offspring well after reaching the terminal moult (Jillison and Edmands, unpublished data). To ensure females used in designed crosses were unmated, we removed clasping males from virgins using a needle probe under a dissecting scope on filter paper. The virgin females were paired with the appropriate males and allowed to mate. After a female was successfully fertilized and the pairs were no longer clasped, the fertilized female was monitored daily for the appearance of an egg sac and then the hatching of larvae. Once larvae were observed, they were counted, and the female was moved to a new dish to allow for subsequent clutch development. Larvae were fed weekly with co-occurring water changes and were allowed to mature and undergo full-sib mating. We acknowledge that mating and reproduction can impact longevity and oxidative damage in both sexes (e.g. [44,56,57]), and chose to assess sex differences in the more natural scenario that includes mating and reproduction. Twenty-eight days after hatching, males were identified by their diagnostic geniculate first antennae commonly referred to as claspers. The sexes were enumerated to estimate the adult sex ratio (hereafter called sex ratio) and separated. Animals were not sexed before adulthood because sexes are difficult to distinguish at immature developmental stages. After the males and females were separated, we monitored each family weekly to determine the number of animals that died until all animals perished. Twenty-eight days and 56 days after hatching, male and female animals were stored by rinsing individuals with water on filter paper and then freezing at −80°C. Archived samples were used to estimate mtDNA content and 8-OH-dG DNA damage.

Survival was fit to a semi-parametric Cox-proportional hazard model with mixed effects [58] and a fully parametric Gompertz model [59]. For the Cox-proportional hazard model with mixed effects, we estimated the interactive fixed effects of sex and cross. Model selection was performed by comparing Akaike's information criterion (AIC) values using the *anova()* function in R v. 3.5.0 [60]. The best fit model included the random effects of family and clutch nested within a family. *Post hoc* testing was performed using least-square means with Tukey adjusted *p*-values.

We fit a two-parameter Gompertz survival model where the family mortality rate (R) at any age (t) can be expressed as

$$R(t) = Ae^{Gt} \tag{2.1}$$

where A represents a theoretical initial mortality rate and G represents the rate of mortality acceleration [61,62]. The Gompertz model was fit for each sex within each family for families that had more than 10 individuals per sex because Gompertz parameter estimation is sensitive to small sample sizes [63]. Gompertz parameters were fit to a linear mixed effects model with random effects of the family to explore the interactive effects of cross and sex [64]. *Post hoc* testing was performed using least-square means with Tukey adjusted *p*-values.

(c) mtDNA content estimation

To extract DNA, individual copepods were incubated for 1 h at 65°C in 50 µl proteinase-K (200 ug ml^{-1}) cell-lysis buffer (10 mM TRIS, 50 mM KCl, 0.5% Tween 20, at pH 8.8) followed by denaturation for 15 min at 100°C.

Using individual DNA lysate, we estimated mtDNA copy number through quantitative polymerase chain reaction (qPCR) for individuals sampled 28- and 56-days post-hatching. To estimate mtDNA content, we targeted single-copy genes for the mitochondrial and nuclear genomes (electronic supplementary material, table S1). We designed primers using Primer-BLAST [65] to target the *AtpC* nuclear gene and the *Atp6* mitochondrial gene. Because SD and FHL mtDNA sequences are highly divergent [46], we performed pairwise sequence alignment [66] (EMBOSS Needle) to generate a consensus sequence upon which we designed mtDNA primers. Primer annealing temperatures were optimized by gradient PCR, and we followed amplification with a melt curve and agarose gel to ensure each primer pair generated a single amplicon. To generate the standard curves for each primer pair, we performed five, 10-fold serial dilutions on DNA extracted from pooled animals. The efficiencies of each primer set were 90–100% with $r^2 > 0.99$ (electronic supplementary material, table S1). The qPCR reaction mixture consisted of 1X Hot-Start ReadyMix (Kapa Biosystems), 1X EvaGreen® Dye (Biotium), 0.5 µM Primers (electronic supplementary material, table S1) and 1 µl DNA lysate. Reaction conditions were as follows: 95°C for 3 min for initial denaturation step, followed by denaturation at 95°C for 15 s, annealing (electronic supplementary material, table S1) for 15 s and extension at 72°C for 20 s repeated 35 times. qPCR reactions were run on a CFX96 Touch real-time PCR detection system (Bio-Rad), and threshold values (Ct) were obtained using CFX Maestro™ Software for CFX real-time PCR instruments (Bio-Rad) using regression. Each reaction was performed in triplicate and the mean was used to calculate mtDNA content in a delta-Ct manner according to Rooney *et al*. [67].

To meet the statistical model assumptions of normality, mtDNA content was log-transformed. Using R v. 3.5.0 [60] and the *lme4* package, we fit data to a linear mixed effects model with random effects of family and clutch within each family [64]. Similar to hazard model fitting, we determined the best fit model using the *anova()* function. After determining the best fit random effects, we estimated the fixed effects of sex, cross, age and their interactions on mtDNA content. *Post hoc* testing was performed using least-square means with Tukey adjusted *p*-values.

(d) Oxidative DNA damage assay

Oxidative stress is the result of the imbalance between total antioxidant defenses and the production of ROS [28]. When more ROS are produced than can be reduced, organisms experience oxidative stress which can damage lipids, proteins and DNA. Here, we estimated DNA damage caused by oxidative stress by measuring 8-OH-dG content; DNA damage which is the result of guanosine oxidation. Samples frozen at 28- and 56-days post-hatching were combined by sex within each family and age class to estimate 8-OH-dG damage using enzyme-linked immunosorbent assay (ELISA; Cayman Chemical cat. 589 320). As determined through serial dilution, the minimum amount of DNA required for the ELISA reaction is 10 ng. To maximize DNA extraction yield, we used a phenol–chloroform extraction technique. First, individuals underwent proteinase K-lysis buffer extraction (see *mtDNA content estimation*). Then, 100 µl of the phenol–chloroform mixture (pH 8.0: VWR) was added to each lysate and the mixture was vortexed and then

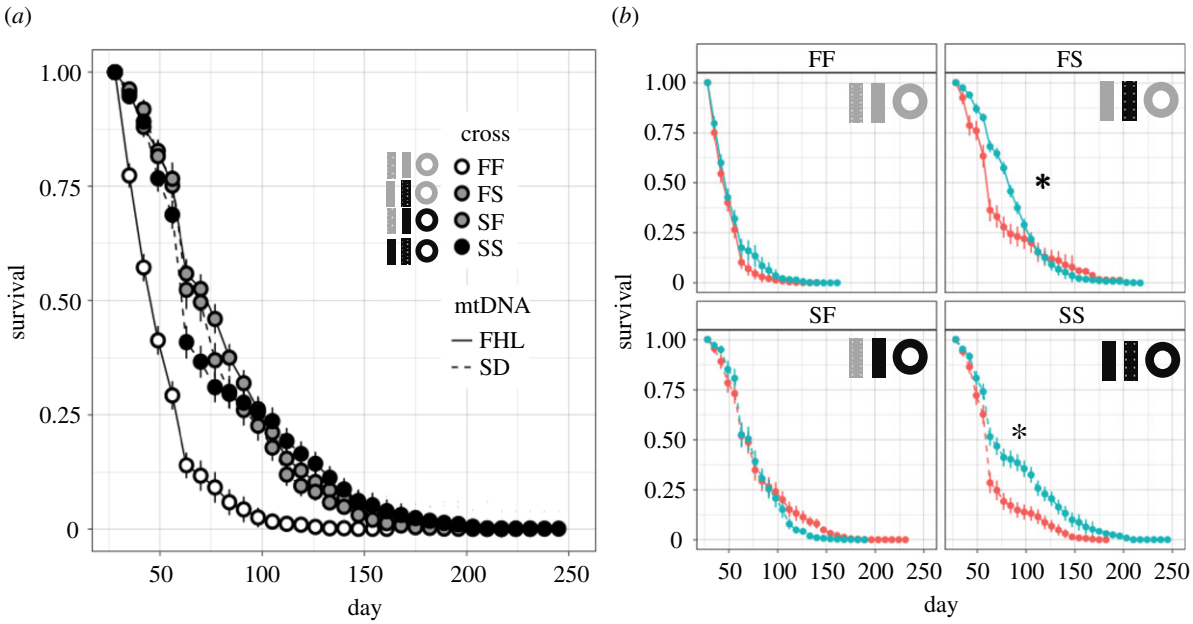

Figure 2. *T. californicus* survival proportions (±s.e.m.) for all crosses (*a*) and for sex differences (male—blue; female—red) within each cross (*b*). Genetic makeup is illustrated in each plot and refers to the cross design outlined in figure 1. Circular colour represents mtDNA genome (black—SD; grey—FHL) while the two vertical bars represent the nuclear genome (black—SD; grey—FHL). Asterisks indicate significant *post hoc* differences ($p < 0.05$) among pairwise estimated marginal means with Tukey corrected *p*-values from the Cox mixed effects model estimating the interactive effects cross and sex (*b*). (Online version in colour.)

centrifuged for 5 min. The aqueous layer was removed and an additional 80 µl of 0.5x TE (pH 8.0) was added and samples were briefly vortexed then centrifuged for 5 min. The second aqueous layer was combined with the first and 500 µl ice-cold 95% ethanol, 1 µl GlycoBlue (Invitrogen) and 78 µl of 3 M NaOAc. The samples were inverted and incubated at −20°C for 1 h and then centrifuged for 30 min. The ethanol was decanted leaving the pellet, which was washed again with 300 µl ice-cold 70% ethanol and centrifuged for 10 min. The ethanol was again decanted, and the remaining DNA was dried by vacuum centrifuge. DNA samples were resuspended in 60 µl molecular grade water and DNA was quantified using a Qubit™ 3 Fluorometer (Invitrogen) using the Qubit™ dsDNA HS Assay Kit (Invitrogen). Samples were then treated with P1 nuclease (New England Biolabs) then rSAP (New England Biolabs), replicate individuals were pooled by sex within each family and age. 8-OH-dG damage ELISA was performed according to the manufacturer's protocol. For each sample, the four technical replicates were averaged, and data were analysed as the ratio of DNA damage to total DNA.

To estimate the effects of age, sex and cross and their interactions on DNA damage, we performed an ANOVA. Data were log-transformed to meet normality assumptions. *Post hoc* testing was performed using least-square means with Tukey adjusted *p*-values.

(e) Line cross analysis and heterosis

To estimate the underlying genetic architecture of the traits measured (longevity, sex ratio, fertility, mtDNA content, 8-OH-dG DNA damage, initial mortality rate and rate of mortality acceleration), we performed a line cross analysis (LCA) using the R package SAGA2 [68] to estimate the composite genetic effects (CGEs) contributing to population trait divergence. The SAGA2 software uses weighted least-square means regression weighted by cohort mean variance to determine phenotypic contribution of CGEs and automatically generates the C-matrix based on breeding design (electronic supplementary material, table S2). Using corrected AICs to explore all models, this method estimates unbiased contribution for each CGE to cohort mean. To determine the relative importance of each CGE contributing to population phenotype divergence, the variable importance is calculated (v_i). Because sex determination in this system is polygenic, we treated sex as a binary environment. A CGE is considered significant if $v_i > 0.9$ and the confidence intervals do not overlap with zero as described in Blackmon & Demuth [68].

Previous work on *T. californicus* F_1 interpopulation hybrids revealed heterosis for a variety of traits including fertility, survivorship and development rate [69,70]. Here, we tested for heterosis (specifically mid-parent heterosis, MPH) for longevity, fertility and mtDNA content using:

$$\mathrm{MPH} = \frac{\bar{F}_1 - \bar{P}}{\bar{P}}, \tag{2.2}$$

where $\bar{F}$ is the mean offspring trait value and $\bar{P}$ is the mean parental trait value [71]. Heterosis was evaluated using linear contrasts with significance level estimated by *t*-tests.

(f) Data manipulation and plotting

All analyses were carried out in R v. 3.5.0 [60] with the use of *dplyr* [72] for data manipulation and *ggplot2* [73] for data visualization.

3. Results

(a) Survivorship and longevity

In total, we observed mortality for 5973 animals which included 35 FF, 26 FS, 16 SF and 27 SS families, each representing biological replicates (figure 1). Mean male longevity was 84.0 ± 0.639 days (±s.d.), while female longevity was 71.5 ± 0.807 days representing a 15.6% mean decrease. Experimental survival data were fit to a Cox mixed effects model. The parental FF cross experienced high mortality early in life resulting in lower longevity when compared to the remainder of the crosses (*coxme*, $\chi^2_3 = 739.99$, $p < 2.2 \times 10^{-16}$). No remaining crosses exhibited differences in survivorship (figure 2*a*). We detected a significant interaction of sex and cross (table 1; electronic supplementary material, table S3A), and *post hoc*

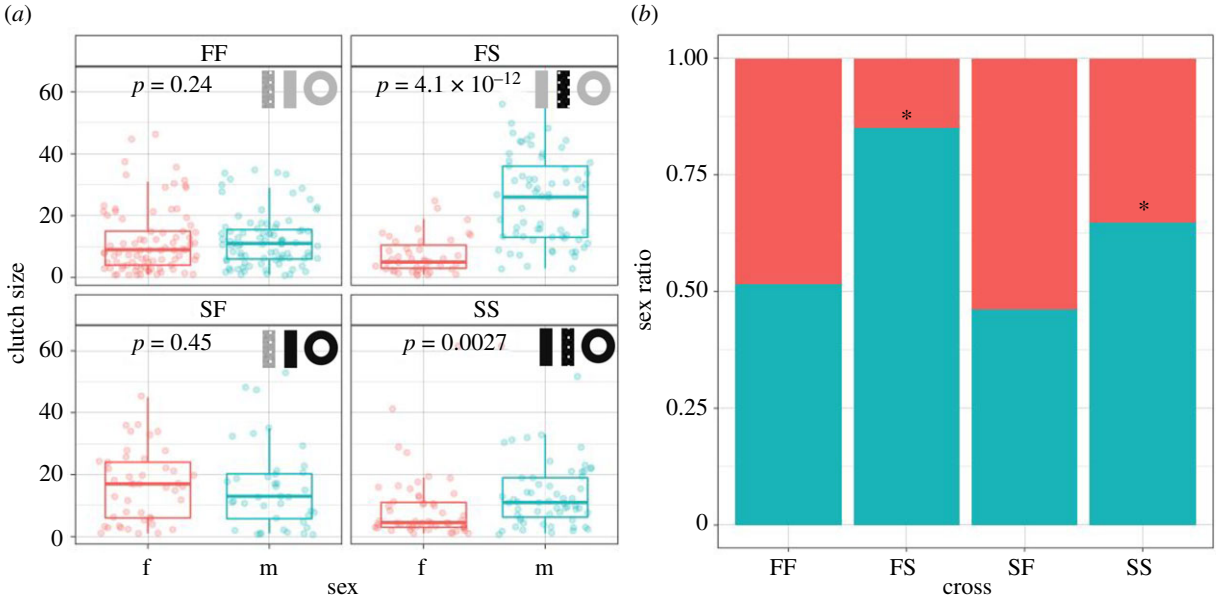

Figure 3. Sex ratio for each cross independently represented as the number of each sex (male—blue; female—red) per clutch (*a*) and the overall proportion for each cross (*b*). *P*-values indicate significance level. Genetic makeup illustrated in (*a*) refers to the cross design outlined in figure 1. Circular colour represents mtDNA genome (black—SD; grey—FHL) while the two vertical bars represent the nuclear genome (black—SD; grey—FHL). Asterisks indicate significance ($p < 0.05$) (*b*). (Online version in colour.)

Table 1. Analysis of deviance table summarizing the interactive effects of sex and cross on survival estimated from a Cox mixed effects model.

	d.f.	chi-sq.	*p*-value
sex	1	14.225	1.622–4
cross	3	739.711	$<2.2 \times 10^{-16}$
sex : cross	3	71.966	9.472×10^{-15}

(electronic supplementary material, table S4) testing indicated the FF cross showed no difference among sexes (log(hazard ratio) = −0.071 ± 0.061, *Z*-ratio = −1.156, $p = 0.944$), while males in the parental SS cross lived longer than SS females (log(hazard ratio) = 0.5612 ± 0.0944, *Z*-ratio = 5.948, $p < 0.001$).

Among the two-hybrid crosses which differ at the mitochondria and the parental source of nuclear contributions, the effect sizes were in opposite directions. For the FS cross with the FHL mitochondria, female FHL contribution and male SD contribution, males lived longer than females (log(hazard ratio) = 0.569 ± 0.086, *Z*-ratio = 6.621, $p < 0.001$). In the reciprocal F1 hybrid with the SD mitochondria, female SD contribution and male FHL contribution, females had a tendency to live longer (log(hazard ratio) = −0.237 ± 0.095, *Z*-ratio = −2.603, $p < 0.154$) (figure 2*b*). Further, linear mixed effects modelling investigating the interactive effects of sex and cross on lifespan corroborated the Cox mixed effects modelling (electronic supplementary material, table S5).

To determine the way sex alters lifespan in *T. californicus*, we assessed the impact of sex and cross on both the initial mortality rate and the rate of mortality acceleration estimated from the Gompertz model (equation (2.1)). For initial mortality rate (electronic supplementary material, figure S5), the rate was lower in males than in females (electronic supplementary material, table S6; table S7; *lmer*, $\chi^2_1 = 10.713$, $p = 1.064 \times 10^{-3}$). Cross had a significant effect (electronic supplementary material, table S6, *lmer*, $\chi^2_1 = 13.4$, $p = 3.846 \times 10^{-3}$) with the FF parental cross showing the highest initial mortality rate and no differences detected among the remaining three crosses (electronic supplementary material, table S7). No interaction among sex and cross was detected for the initial mortality rate (electronic supplementary material, table S6; *lmer*, $\chi^2_3 = 0.878$, $p = 0.831$). For the rate of mortality acceleration (electronic supplementary material, figure S5), the main effects of sex (electronic supplementary material, table S8; *lmer*, $\chi^2_1 = 5.308$, $p = 0.021$) and cross (electronic supplementary material, table S8; *lmer*, $\chi^2_3 = 36.646$, $p = 5.469 \times 10^{-8}$) and their interaction (electronic supplementary material, table S9; *lmer*, $\chi^2_3 = 7.66$, $p = 0.054$) tended to impact the rate of mortality acceleration. *Post hoc* testing indicated males from the FF cross have the highest rate of mortality acceleration when compared to the other sexes and crosses (electronic supplementary material, table S7).

(b) Sex ratio

The sex ratio was male-biased in two of the four crosses: the parental SS cross and the FS hybrid cross with the FHL mitochondria (figure 3*a*,*b*; *t-test*, $p < 0.05$). If the male-biased sex ratio was driven by sex-biased survivorship during development, we would expect crosses FS and SS to show a positive association between the proportion of males assigned at day 28, and the number of individuals that died during development from hatching through larval and copepodite moults to sexual maturity and assignment of sex at day 28. Instead, we found no correlation for crosses FS, SS and SF, and a negative correlation for cross FF (electronic supplementary material, figure S1).

(c) MtDNA content

Sex had a strong effect on mtDNA content (electronic supplementary material, table S10A, *lmer*, $\chi^2_3 = 23.1$, $p = 1.54 \times 10^{-6}$), with mean mtDNA copy number in females (182 ± 13.4) being greater than in males (121 ± 11.6). No cross effect was observed. Age and sex effects tended to interact (electronic supplementary material, table S10A, *lmer*, $\chi^2_3 = 3.07$, $p = 0.079$) and *post hoc* testing revealed male

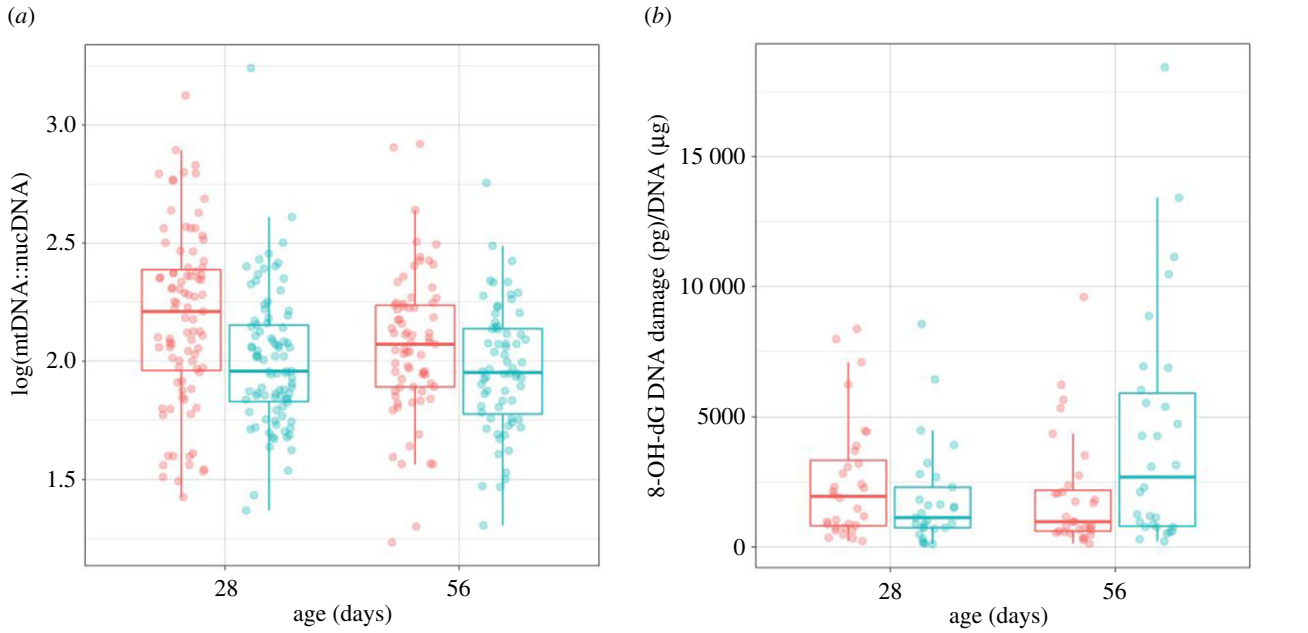

Figure 4. Mitochondrial DNA content for male and female *T. californicus* 28- and 56-days post-hatching (*a*). 8-OH-dG DNA damage estimated by ELISA for two ages and each sex (male—blue; female—red) (*b*). (Online version in colour.)

mtDNA content is invariant with age while as females age, their mtDNA content decreases. Further, 28 days after hatching, males had lower mtDNA content than females (figure 4*a*; electronic supplementary material, figure S3; table S11).

(d) 8-OH-dG DNA damage

Sex and age interactively impacted 8-OH-dG DNA damage (figure 4*b*; electronic supplementary material, figure S4; table S12). For males, DNA damage increased with age to the point where 56 days post-hatching male damage tended to be higher than female damage (electronic supplementary material, table S13).

(e) Line cross analysis and heterosis

To estimate the sex-specific genetic architecture of longevity, an LCA was performed where sex was treated as an environmental condition. Following Blackmon & Demuth [68], CGEs were considered significant if they had a variable importance (v_i) greater than 0.9 and a parameter estimate where the confidence interval excluded zero. For longevity, autosomal additive variation significantly contributed to population trait divergence where the confidence intervals did not overlap zero with $v_i = 0.976$ while all other CGEs, including those with the sex term, had v_i less than 0.5 (figure 5). For sex ratio, the epistatic interaction of autosomal additive variation with cytoplasmic additive variation significantly contributed to the population divergence in sex ratio where $v_i = 0.91$ (figure 5) indicating a mitonuclear interaction for sex ratio. As for mtDNA content, the sexual state treated as an environment significantly contributed to the variation in mtDNA where we observed a $v_i = 0.95$ (figure 5). The only significant genetic effect detected contributing to DNA damage was the epistatic interaction of autosomal dominant variation interacting with the environment of sex $v_i = 0.979$ and non-zero overlapping confidence intervals. Both autosomal additive variation and variation due to the environment of sex significantly contributed to the initial mortality rate (electronic supplementary material, figure S2). No significant CGEs were detected for fertility and the rate of mortality acceleration.

Both SF and FS F1 hybrid crosses showed heterosis for clutch size and longevity, but not mtDNA content (electronic supplementary material, table S14).

4. Discussion

This study represents the first large-scale estimation of sex-specific lifespan in this species. Here, we find autosomal nuclear variation primarily contributed to the variation in longevity, yet by using F1 hybrids which share a 50 : 50 nuclear background but differ at the mitochondria and sex-specific parental contributions, and by comparing parentals to hybrids, we detected a mitonuclear effect for sex-specific longevity. The FS hybrid cross showed male-biased longevity, yet no sex difference was detected for the reciprocal SF hybrid which differs at the mitochondria and the parental source of nuclear contributions. Male-biased longevity was also observed in the parental SS cross indicating sex differences in longevity depend both on nuclear and mitochondrial genotype. This represents a mitonuclear interaction for sex-specific ageing. In those crosses with male-biased longevity (FS and SS), we also observed a male-biased sex ratio. The only significant genetic element that contributed to the sex ratio divergence was the epistatic interaction of autosomal and cytoplasmic variation, indicating the observed male-biased sex ratio is a result of mitonuclear interactions.

Males were found to live longer than females in two of the four crosses, which was unexpected because *T. californicus* males are less tolerant to a variety of abiotic stressors [50], and stress tolerance in other taxa is often positively associated with lifespan [74,75]. There is even evidence for a causal relationship between stress tolerance and longevity, with selection for increased stress resistance resulting in increased lifespan [76] and vice versa [77]. The two crosses sired by the San Diego population showed male-biased longevity and male-biased sex ratio. The change in sex ratio was attributed

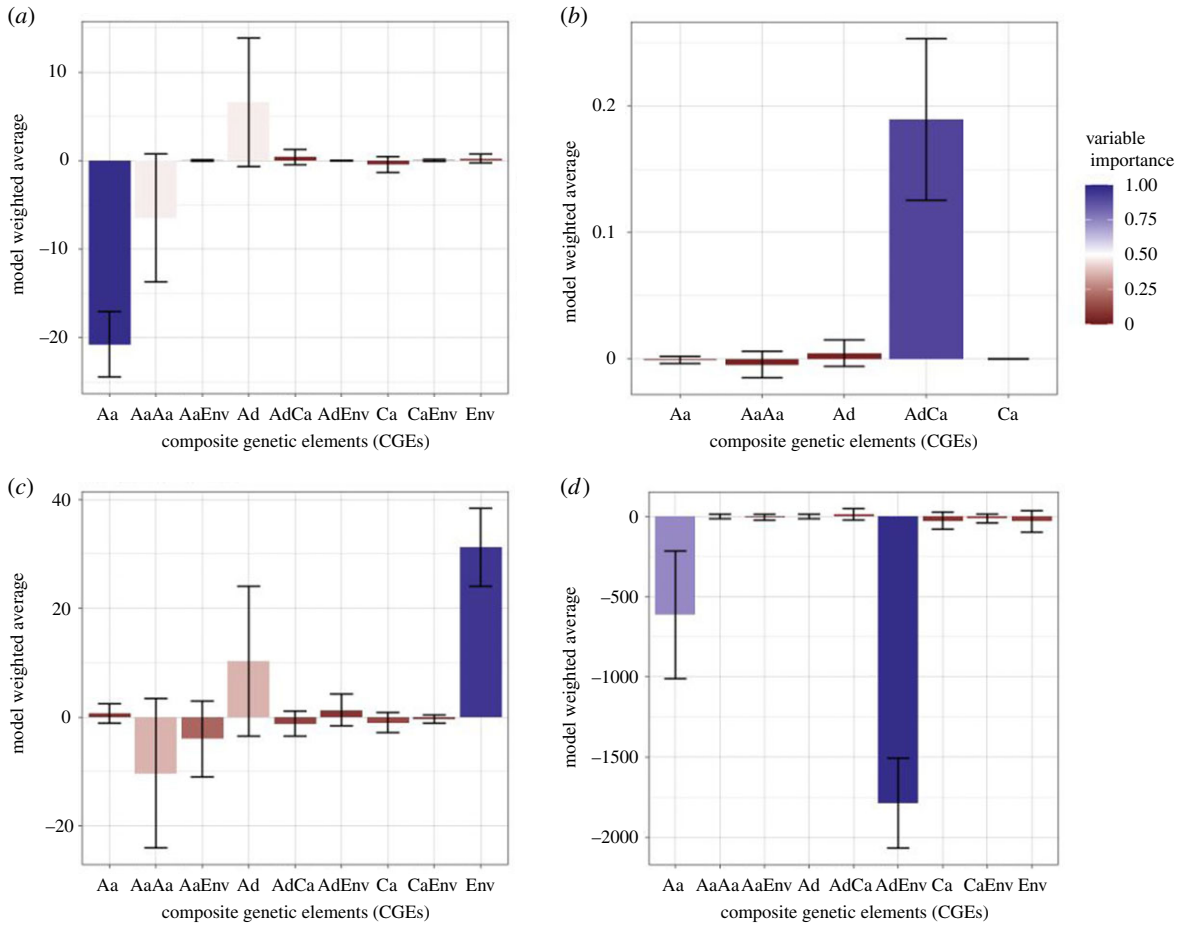

Figure 5. LCA model weighted average of parameter estimates for mean longevity (*a*), sex ratio (*b*), mtDNA content (*c*) and DNA damage (*d*). Significant CGEs are autosomal additive (Aa), autosomal dominant by cytoplasmic additive epistasis (AdCa) and autosomal additive by environment interactions (AaEnv). Bars represent mean model weighted averages and error bars are unconditional standard errors (see Blackmon and Demuth [68]) while colours represent relative variable importance (v_i). (Online version in colour.)

to mitonuclear interaction, but the LCA models could not detect a sex effect because sex ratio is a composite male/female trait. The sex ratio differences could be the result of sex-specific developmental mortality, but the absence of a correlation between sex ratio and mortality during development in the two male-biased crosses argues against this hypothesis. An alternative explanation is the presence of a sex ratio distorter, as suggested by [49]. While, sex-specific effects in this system are not complicated by the effects of sex chromosomes [47–49], inferring mitochondrial effects in F1 hybrids may be confounded by sex-specific paternal genetic or non-genetic contributions which differ between reciprocal hybrid crosses. Under polygenic sex determination, multiple portions of the genome contribute to sex and those genomic regions could contribute to sexually dimorphic life-history traits in this species.

The relative differences in longevity between males and females may depend on lifetime energy expenditure. *T. californicus* females can produce more than 10 egg clutches over their lifespan [78,79] and as females age, they produce smaller clutches in terms of quantity and size [79]. Here, we find young females had the highest mtDNA content and as females aged, mtDNA content decreased while male mtDNA content remained invariant irrespective of the age or the cross. Further, sex primarily contributed to the variation in mtDNA content. This suggests that metabolic demands differ among the sexes and the decrease in mtDNA content with age in females may be related to reproductive senescence. In *Drosophila*, longitudinal mtDNA content decline in males was associated with reduced stress tolerance later in life [36]. The decrease in female mtDNA content with age may represent increased mitochondrial malfunction resulting from sex-specific energetic demands. Age associated stress tolerance decline has not been documented in this species yet may give insight into the relationship between female mtDNA content decline and aged physiologies.

Hormetic effects on lifespan are characterized by exogenous stress exposure resulting in lifespan extension [80–82], and these effects are not consistent across sexes where one sex is more responsive to hormetic intervention and thus show a greater increase in lifespan [81,83]. ROS-induced oxidative DNA damage increased with age in males and was greater in males 56 days after hatching. If the measured DNA damage is representative of endogenous ROS levels, male and females may experience different levels of oxidative damage throughout their lives. In *T. californicus*, gene expression was more greatly affected by sex differences than exogenous oxidant exposure, where male copepods differentially expressed more genes than females [51,52], including the upregulation of antioxidant associated genes. Elevated endogenous ROS levels could result in the male-specific hormetic response especially if the ROS is mitochondrially derived. Schaar *et al*. [84] found cytoplasmic ROS negatively impacted lifespan while ROS localized to the mitochondria extended lifespan in *Caenorhabditis elegans*. The mechanism leading to increased male lifespan in

T. californicus remains unclear, yet we postulate it occurs due to ROS-induced mitohormesis. The hypothesized mechanism could be tested by assessing the response of lifespan to experimental manipulation of ROS (e.g. [85]).

Ultimately, extrinsic mortality rates shape the evolution of ageing [86], and the sex that experiences the higher extrinsic mortality is predicted to have a shorter life [5]. Therefore, longevity represents a trade-off between mortality and reproduction [5,57] and selection can act upon the trade-off resulting in sex-specific life-history optima [87,88]. If we consider the *T. californicus* mating system where females only mate once and continually produce offspring throughout the lifespan [56,79], while males can mate multiply, the senescent reproductive decline may negatively impact males more than females. If male mating success fails to decrease with age, selection may favour increased lifespan in males [89,90]. While males do live longer than females in half of the crosses in this study, we did not determine the reproductive value of males as we only characterized mortality. Estimation of the reproductive value of males and females may elucidate the selective pressures potentially leading to lifespan dimorphism observed in *T. californicus*.

One hypothesis which describes the evolution of sex-specific mitochondrial effects is the Mother's Curse [10,11]. Under the hypothesis, the prediction is decreased male lifespan [14,91] which was not supported by our study. Another prediction (e.g. [92]) is that mitonuclear mismatch may cause more fitness-related problems in males than in females, yet recent work in *Drosophila* failed to detect this hypothesized effect [93]. Our study is not well suited to test this prediction as the F1 hybrids did not show fitness problems instead the reciprocal crosses both showed hybrid vigor for lifespan, clutch size and mtDNA content.

In sum, sex differences in *T. californicus* longevity depended upon mitochondrial and nuclear genotype, and the male-biased sex ratio was the result of mitonuclear effects. mtDNA content decreased in females which may be an indication of senescence related to mitochondrial dysfunction while males showed an increase in DNA damage with age. One hypothesis for the longevity dimorphism is the sex-specific action of mitohormesis in males. This is consistent with the findings of accelerated accumulation of oxidative damage in males but would require further experimentation to confirm. In this system where mitochondrial effects are not confounded by sex chromosomes, the comparison of reciprocal F1 hybrids and parental crosses revealed sex differences in longevity were the result of mitonuclear interactions.

Data accessibility. The data and code supporting this paper are available from Zenodo: https://doi.org/10.5281/zenodo.5177219 [94].

Authors' contributions. B.A.F.: data curation, formal analysis, investigation, methodology, validation, visualization, writing-original draft, writing-review and editing; N.L.: data curation, investigation, methodology, validation, writing-review & editing; S.E.: conceptualization, funding acquisition, project administration, supervision, writing-review and editing.

All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

Competing interests. We declare we have no competing interests.

Funding. This work was supported by the National Institute on Ageing of the US National Institutes of Health (R21AG055873 awarded to S.E.) and the US National Science Foundation (DEB-1656048 awarded to S.E.).

Acknowledgements. We would like to thank Dr Heath Blackmon for discussion implementing LCA in SAGA2 and Dr Felipe Barreto with assistance in ELISA methodology. Thanks to Dr Eric Watson, Dr Scott Applebaum, Alice Coleman and two anonymous reviewers for input which improved an earlier version of this manuscript.

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