ORIGINAL ARTICLE

Direct and indirect genetic effects of sex-specific mitonuclear epistasis on reproductive ageing

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Mitochondria are involved in ageing and their function requires coordinated action of both mitochondrial and nuclear genes. Epistasis between the two genomes can influence lifespan but whether this also holds for reproductive senescence is unclear. Maternal inheritance of mitochondria predicts sex differences in the efficacy of selection on mitonuclear genotypes that should result in differences between females and males in mitochondrial genetic effects. Mitonuclear genotype of a focal individual may also indirectly affect trait expression in the mating partner. We tested these predictions in the seed beetle *Callosobruchus maculatus*, using introgression lines harbouring distinct mitonuclear genotypes. Our results reveal both direct and indirect sex-specific effects of mitonuclear epistasis on reproductive ageing. Females harbouring coadapted mitonuclear genotypes showed higher lifetime fecundity due to slower senescence relative to novel mitonuclear combinations. We found no evidence for mitonuclear coadaptation in males. Mitonuclear epistasis not only affected age-specific ejaculate weight, but also influenced male age-dependent indirect effects on traits expressed by their female partners (fecundity, egg size, longevity). These results demonstrate important consequences of sex-specific mitonuclear epistasis for both mating partners, consistent with a role for mitonuclear genetic constraints upon sex-specific adaptive evolution.

INTRODUCTION

Ageing (senescence) is a near-universal characteristic of living organisms and is manifested as decreasing performance and increasing probability of death with age due to physiological deterioration (Monaghan *et al.*, 2008). Mitochondrial malfunction has been implicated in ageing across species and is apparent as structural abnormalities of mitochondria, decreased electron transport chain enzyme activity in the oxidative phosphorylation pathway, increased production of reactive oxygen species and accumulation of mutations on mitochondrial DNA (mtDNA) (Tower, 2014). Mitochondrial function relies on the coordinated expression of over a thousand nuclear genes and a few dozen mitochondrial genes (Calvo and Mootha, 2010). This suggests that ageing phenotypes may commonly be subject to a complex set of epistatic interactions between the nuclear and the mitochondrial genomes. Mitonuclear interactions affect lifespan through redox balance involving nuclear-encoded antioxidants that defend against oxidative stress (Finkel and Holbrook, 2000), retrograde signalling (Liu and Butow, 2006) and mitonuclear protein imbalance via activation of the mitochondrial unfolded protein response (Houtkooper *et al.*, 2013).

Some studies have found effects of mitochondrial polymorphism or mitonuclear epistasis on longevity and survival (Maklakov *et al.*, 2006; Rand *et al.*, 2006; Camus *et al.*, 2012; Zhu *et al.*, 2014) but their role in reproductive ageing is less well studied (Yee *et al.*, 2015). The influence of mitochondria in general, and of mitonuclear interactions in particular, may be even larger for reproductive senescence compared with actuarial ageing (that is, longevity and survival) (Maklakov and Lummaa, 2013; Dobler *et al.*, 2014). This is partly because reproduction is sensitive to cellular malfunctioning, and there is a general decline in mitochondrial performance with age (Lee and Wei, 2012; Tower, 2014), but also because sexual asymmetries in inheritance and selection should interact to sustain mitonuclear epistasis. Males and females often optimise their reproductive scheduling in distinct ways and selection should thus generate sex differences in the pattern of reproductive and actuarial ageing (Bonduriansky *et al.*, 2008). Such sex-specific selection predicts that mitonuclear genetic effects on the pattern of ageing may also differ between males and females. For example, the involvement of mitochondria places evolutionary constraints on male-specific optimisation of reproduction and somatic maintenance, because mtDNA can only respond to selection acting on females. This represents an irreconcilable form of intralocus sexual conflict (Bonduriansky and Chenoweth, 2009) that can lead to the accumulation of a male-specific mutation load, a phenomenon known as Mother's curse (Gemmell *et al.*, 2004). One of the key predictions of the Mother's curse is that male-harming mitochondrial mutations should select for counteradaptations in nuclear genes interacting with the mitochondria, leading to mitonuclear coevolution (Gallach and Betran, 2011; Wade, 2014). However, it has been estimated that the rate of nuclear compensatory evolution is less than one-fourth that of male-harming mitochondrial mutations (Wade, 2014), making mitonuclear coevolution less efficient in males. Concordantly, many studies on male-specific mitonuclear effects find irregular epistasis rather than increased mitochondrial performance in coadapted mitonuclear combinations

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(Dobler et al., 2014). In females, selection on mitonuclear genotype would be more efficient in terms of generating mitonuclear coadaptation, simply because nuclear genes and mtDNA cosegregate in females (Rand et al., 2004; Wade, 2014). Sex-specific mitonuclear coevolution may also be constrained by sexually antagonistic selection on the nuclear genes (Rand et al., 2001). Although mitonuclear epistatic effects have been demonstrated in both sexes, very few studies have studied both sexes simultaneously (Dobler et al., 2014).

An important but overlooked aspect in the study of sex-specific reproductive senescence derives from the fact that reproductive traits in one sex, affected by mitochondrial genetic variation (Dowling et al., 2007b; Yee et al., 2013), may have important effects on reproductive phenotypes in the other sex. Reproduction is an outcome of molecular interactions between the sexes and research over the past few decades has clearly demonstrated that male traits influence female reproductive performance (Arnqvist and Rowe, 2005). It is thus possible that mitochondrial genetic effects may extend beyond focal males (that is, direct genetic effects) to also affect their mating partners (that is, indirect genetic effects) (Moore et al., 1997). Such effects are most likely mediated by seminal fluid proteins that are transferred to females during mating, with manifold effects on female reproduction (Avila et al., 2011). Male reproductive senescence could thus influence female traits via male seminal fluid biosynthesis and could be affected by mitonuclear epistasis through the role that mitochondria play in metabolic senescence.

Here, we explicitly test for mitonuclear epistasis on several reproductive and life-history characteristics of males and females throughout their life, using Callosobruchus maculatus seed beetles with distinct mitonuclear genotypes created by introgression between naturally occurring mtDNA haplotypes and nuclear genotypes from three populations. Our main objectives are to test the following predictions: (1) mitonuclear epistasis influences reproductive senescence, (2) such effects are sex specific and show differences regarding mitonuclear coadaptation and (3) mitonuclear genetic variation in males is associated with indirect genetic effects on traits expressed by their mates.

MATERIALS AND METHODS
Study organism
The seed beetle C. maculatus occurs in subtropical and tropical regions throughout the world, and is a major pest of legume plants. Females lay eggs on beans and the first instar larvae bore into them within 2–4 days. The larvae develop for ~3 weeks, and copulate soon after emergence from beans as adults. Both sexes can mate multiply. This species is facultatively aphagous and can complete its reproductive cycle with the resources acquired during the larval stage (Fox, 1993). However, adult feeding increases both longevity and productivity of the beetles (Fox, 1993). Previous work has documented mitonuclear genetic effects on basal metabolic rate in pupae (Arnqvist et al., 2010) and in mated males (Immonen et al., 2015), juvenile growth rate (Dowling et al., 2007a), behavioural syndromes (Lovlie et al., 2014) and sperm characteristics (Dowling et al., 2007b).

Mitonuclear lines
In order to disentangle the effects of mitochondrial and nuclear genetic variation as well as their epistatic interaction we introgressed three distinct naturally occurring mitochondrial genomes into three nuclear backgrounds. Sequencing of two mitochondrial genes (1005 bp of cytochrome oxidase subunit I (COI) and 473 bp of cytochrome b (Cyt-b)) has documented substantial genetic variation across these three mtDNA haplotypes (Arnqvist et al., 2010). Approximately 2–3% of all mtDNA sites are polymorphic, and some sites show nonsynonymous differences. A detailed description of the construction of the introgression lines can be found in Kazancioglu and Arnqvist (2013). Briefly, three outbred populations of C. maculatus (Brazil

(BRA), California (CA) and Yemen (YEM)), which carry distinct mtDNA haplotypes (Kazancioglu and Arnqvist, 2013), were used to generate nine fully crossed combinations of distinct cytoplasmic and nuclear lineages. These populations were selected from a larger set of potential populations as previous research has shown that mitochondrial genetic variation across these particular populations affects life-history phenotypes (Dowling et al., 2007a,b; Arnqvist et al., 2010; Immonen et al., 2015). A single randomly selected virgin female from each of the populations (ensured to carry a distinct mtDNA haplotype by sequencing, see above) was mated to a male from the same population, and the full-sib daughters of these 'mitochondrial Eves' were subsequently mated to males from one of the three populations. This orthogonal scheme resulted in nine possible mitonuclear combinations, and each particular cross was replicated twice starting with a different female (carrying the same mtDNA haplotype) (that is, total of 18 introgression lines) to allow assessment of variation within mitonuclear combination. Within each cross, virgin daughters in every generation were backcrossed to virgin males from their father's population, thus disassociating the mitochondrial genome from the nuclear genome with which it was originally coexpressed. Backcrossing was repeated for 16 generations, replacing 99.998% of the original nuclear genome with the paternal nuclear genome. Replication of cross types (see above) allowed us to assess effects of this incomplete replacement of the nuclear genome. Fertility, hatching rate and adult viability were not depressed during introgression. Subsequently, the 18 introgression lines were maintained as separate populations in large glass jars seeded with 100 g of Vigna unguiculata black-eyed beans at 29 °C and 12:12 h light/dark cycle with 50% relative humidity. The beetles were maintained under aphagy and the lines were backcrossed once again to the outbred stock populations at generation 17. We validated the mitochondrial integrity of the introgression lines immediately after our experiments by amplification and sequencing of a diagnostic mtDNA marker (COI), following Kazancioglu and Arnqvist (2013). Potential cytoplasmic endosymbionts (for example, Wolbachia) that could confound cytoplasmic effects have carefully been screened for in many C. maculatus populations, including the ones used here, but have never been detected (see, for example, Tuda et al., 2006). Nevertheless, we treated all introgression lines with an antibiotic treatment between generations 13 and 14 (see Kazancioglu and Arnqvist, 2013 for details) to preclude the possibility that the lines may have harboured cytoplasmic bacterial infections. Moreover, mtDNA sequence divergence across these haplotypes is quantitatively associated with phenotypic divergence across haplotypes (Arnqvist et al., 2010).

Experimental procedures
The reproductive performance of the males and females from the introgression lines were recorded by mating them to individuals from an outbred standard reference population (SI USA) at fixed intervals through their reproductive life. The reference population was maintained on mung beans (Vigna radiata) under the same abiotic condition as the focal introgression lines. Virgin focal and reference individuals were collected by isolating beans with larvae in chambers from which hatching adults were collected. Virgins were then isolated in 1.5 ml Eppendorf tubes (with lids punctured for air) until used in the experiments.

To ensure that beetles would not become dehydrated or resource limited with age, which could result in starvation effects rather than senescence in physiological performance, we provided both focal and reference individuals food ad libitum throughout their lifespan (baker's yeast and 5% sucrose solution; renewed every 4 days) (Arnqvist et al., 2010).

Females. To characterise the pattern of senescence in females from the introgression lines, we recorded age-specific fecundity ($N=912$), fertility (the proportion of hatched eggs, $N=912$), larval survival (the proportion of emerged adult offspring out of all the hatched eggs, $N=629$) as well as longevity ($N=165$). To ensure the females would not become sperm limited over time, we allowed them to mate for up to four times (to reference virgin males of age 24–48 h) at fixed intervals (Figure 1). We observed all matings and separated the pairs immediately after copulation. After the first mating (at the age of 48 h), the females were allowed to individually lay eggs on petri dishes with an excess of black-eyed beans as an egg-laying substrate, for a fixed interval, after which they were either first provided a remating opportunity or
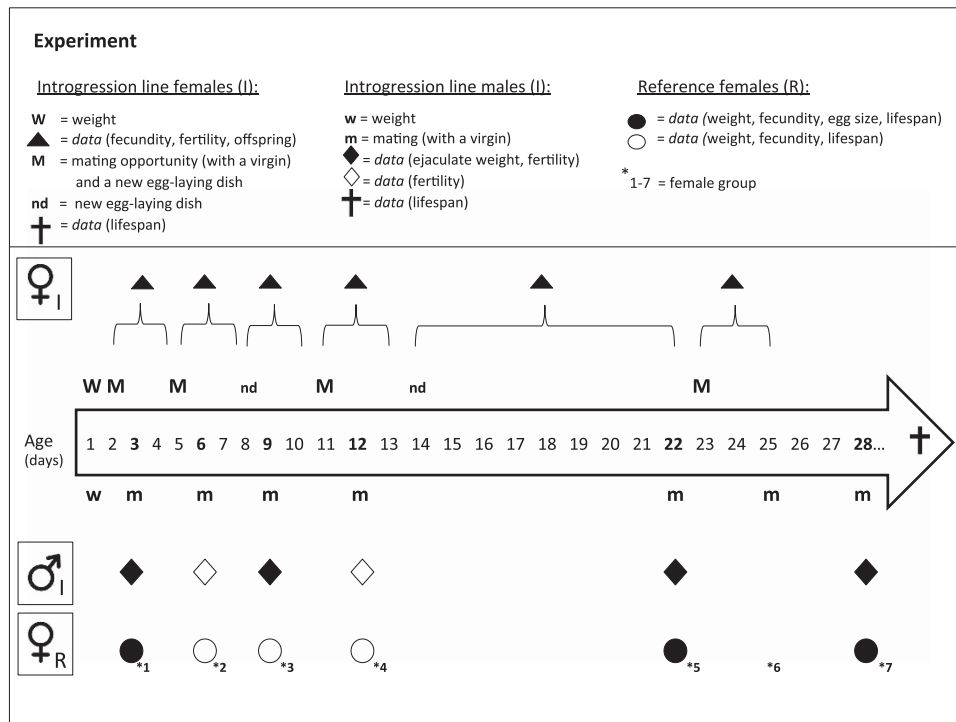
Figure 1 Summary of the experimental design. We recorded data of age-specific reproductive performance and lifespan from mitonuclear introgression lines (I) and a reference population females (R) mated to the introgression line males. For the focal females, provided with a mating opportunity with four sets of reference virgin males (1–2-day-old virgin), we collected data from six age intervals. For the focal males and their reference female mates (1–2-day-old virgin), we collected data from six male age points. The symbols indicate data collected from each experimental group at each time point (open and closed symbols show which variables were recorded at a given time).

directly transferred into a new egg-laying dish (Figure 1). Altogether, we recorded female reproduction from six age intervals, capturing the entire reproductive age span from 2 to 26 days. We counted the hatched and unhatched eggs from each dish after ensuring that all eggs had sufficient time to hatch, and subsequently allowed the eggs to develop into adult offspring. For the post-reproductive period (age 26 days onwards) we transferred the females individually into dishes (with only a few beans and food) where their longevity was recorded. The females were weighed upon emergence to the nearest 0.00001 g (Sartorius AG, Goettingen, Germany) to account for the effect of weight on fecundity and longevity.

Males. We characterised effects of senescence in males from the introgression lines on both traits expressed by themselves and their reference female mating partners (that is, direct and indirect genetic effects). The direct effects measured in males included ejaculate weight ($N = 615$) (measured as the difference in body mass before and after mating, Edvardsson and Tregenza, 2005) and male longevity ($N = 173$).

The indirect effects of male age and genotype on the expression of female traits were measured for fecundity ($N = 621$), egg size ($N = 7971$) and fertility of the eggs (the proportion of hatched eggs, $N = 954$). We also recorded longevity of the female mates ($N = 945$).

We mated males seven times to 24–48 h-old virgin reference females at fixed intervals (male reproductive age span from 3 to 28 days, Figure 1). We observed every mating and separated the pairs immediately after copulation. Controlling mating rate allowed us to focus on effects of male physiological senescence and its consequences on his reproductive performance in the context of mating, without confounding effects of variation in male harassment or mating frequency. After mating, the reference females were kept individually in petri dishes, with an excess of mung beans, for 18 days (to allow plenty of time for all the eggs to hatch but no adult offspring), after which the females were transferred in isolation to new petri dishes (with food and a few beans) where their longevity was recorded. The reference females and focal males were

weighed to the nearest 0.00001 g (Sartorius Genius ME 235P). We measured the ejaculate weight from four matings (ages 3, 9, 22 and 28 days, Figure 1). To measure egg size, we randomly sampled 20 eggs per female, from three matings of male ages 3, 22 and 28 days (Figure 1), and photographed them using a Lumenera (Infinity Technologies, Ottawa, ON, Canada) digital camera mounted on a dissection microscope (Leica Microsystems GmbH, Wetzlar, Germany; MZ8). Egg width, as a proxy for egg size, was measured using ImageJ (v. 1.48; Schneider et al., 2012).

Statistical analyses

The crossed design allows the use of linear effects mixed models to evaluate our results. All of our inferential mixed effects models contain a random effect structure that takes the mitonuclear line replication into account (that is, by fitting the line as a random factor) and, where necessary, the repeated measures taken across age (that is, by fitting individual ID as a random factor). Models were fitted with R (version 3.0.1, see package information below) (R Development Core Team, 2011). All models include mitochondrial lineage ('mito'), nuclear lineage ('nuc') and their interaction as factorial fixed effects, and weight at emergence (of the focal individual) as a covariate.

The significance of terms in the models was assessed with type III sums-of-squares tested with analyses of deviance based on the χ^2 distribution, as implemented in the *car* package (version 2.0–20) (Fox and Weisberg, 2011). Survival data were analysed using Cox proportional hazard mixed models, where the significance of terms was determined by model comparisons after model reductions using the χ^2 tests. Covariates in all models were standardised to a mean of zero and unit variance, with the exception of female age that was only mean centred. Model fit was validated by visual inspection, and few observations with absolute values of the standardised residuals exceeding 3.0 were deemed as outliers an omitted. We note that, in any such occasion, the results remained qualitatively the same even if including these outliers. For the binomial models we estimated overdispersion as the sum of squared Pearson's

Table 1 Direct effects of age, mitochondrial and nuclear genetic variation on introgression line for female fecundity (that is, daily rate of egg laying) and fertility, and male ejaculate weight

Response variable	Fecundity[a], n = 912					Fertility[a], n = 912					Ejaculate weight[b], n = 615				
	Coef	s.e.	χ^2	d.f.	P	Coef	s.e.	χ^2	d.f.	P	Coef	s.e.	χ^2	d.f.	P
Fixed effects															
Age	−0.67	0.04	305.0	1	**<0.0001**	−0.81	0.11	52.5	1	**<0.0001**			5.8	3	0.1198
Body weight	0.16	0.02	97.0	1	**<0.0001**	0.01	0.08	0.002	1	0.9636	5.44	0.40	183.6	1	**<0.0001**
Mating rate	0.04	0.02	6.8	1	**0.0091**	0.13	0.08	2.9	1	0.0874	—	—	—	—	—
Mitochondrial lineage			7.3	2	**0.0266**			1.8	2	0.3929			13.9	2	**0.0010**
Nuclear lineage			6.6	2	**0.0373**			0.0	2	0.9979			4.6	2	0.0986
Mito × Nuc			12.5	4	**0.0141**			8.1	4	0.0887			24.8	4	**<0.0001**
Age × Mito			4.4	2	0.1123			0.3	2	0.8724			31.7	6	**<0.0001**
Age × Nuc			6.0	2	**0.0495**			2.4	2	0.2964			24.7	6	**<0.0001**
Age × Mito × Nuc			10.0	4	**0.0422**			2.3	4	0.6766			36.4	12	**<0.0001**
Random effects	Variance (σ^2)					Variance (σ^2)					Variance (σ^2)				
Line	2.061×10^{-9}					0.00					0.08				
ID	2.066×10^{-9}					0.40					1.08				

Abbreviations: Coef, coefficient; d.f., degrees of freedom; Mito, mitochondrial lineage; Nuc, nuclear lineage. $P < 0.05$ highlighted in bold.
[a]Female.
[b]Male.

residuals divided by the residual degrees of freedom and, if overdispersed, we accounted for this by including an individual observation-level random effect into the model (Elston et al., 2001) and validated the fit with a model comparison using Akaike information criterion and log-likelihood information. Below we present the model details specific to the analyses for each sex.

Females. We examined effects on the rate of female reproductive senescence with the following models. We first investigated effects on fecundity by fitting a zero-inflated negative binomial mixed model (ZINBMM, package 'glmmADMB' version 0.8.0; Fournier et al., 2012), with age as a covariate and its two- and three-way interactions with the genetic factors. Because one of the egg-laying intervals was longer than others (see Figure 1), we used daily fecundity as the response variable, calculated by dividing the number of eggs with the number of days within each interval. The best model fit, based on Akaike information criterion information, was achieved with 'NB1' parametrisation (variance = $\varphi\mu$). Based on the findings of this model (Supplementary Figure S1) we also tested whether an observed mitonuclear × age interaction follows a pattern of mitonuclear coevolution, with an otherwise similar model (see above and Table 1) but replacing the 'mito' factor with a two-level factor representing the mitonuclear coevolutionary history (that is, potentially coadapted or not). Both models also include female lifetime mating rate as a covariate (we note that female remating rate did not differ between the genotypes, result not shown). We tested for effects on fertility and on larval survival with generalised linear mixed models (GLMM, package 'lme4', version 1.1–7; Bates et al., 2014) with the same model structure as for the main inferential model for fecundity, but with an additional observation-level random factor to account for a slight overdispersion.

We examined effects on female actuarial ageing by testing for effects on longevity, with a linear mixed model (LMM, package 'lme4'), including the genetic effects as factors, weight and lifetime fecundity as covariates ($N = 165$). We also tested for survival differences using the Cox proportional hazard mixed model (CPHMM, package 'coxme' version 2.2–3; Thernau, 2012), with the same explanatory variables.

Males. We first assessed direct genetic and age effects on the two reproductive traits expressed by the males. We tested how male ejaculate weight ($N = 615$) varies with age and genotype with a LMM and how these affect male fertility ($N = 954$), using a GLMM, with age fitted as a factor. We also tested whether observed mitonuclear × age interactions confirm with mitonuclear coadaptation, with otherwise similar models but replacing the 'mito' factor with a two-level factor representing coevolutionary history. Observation-level random factor was included in the fertility models to account for overdispersion.

To examine mitonuclear genetic effects on male actuarial ageing, we tested how males vary in longevity with a LMM and in survival using a CPHMM ($N = 173$).

Our second aim was to assess indirect genetic and age effects of males on traits expressed by their female mating partners. We tested for effects on female fecundity with a LMM. As male ejaculate weight was clearly an important determinant, we here only present the model on the subset of the data where we have this information ($N = 621$, Figure 1). We note, however, that genetic and age effects were independent of the ejaculate weight effects. We also explored the effects of mitonuclear coadaptation in the same manner as described above. We tested for male effects on female egg size ($N = 7971$) with a LMM, including the reference female ID as an additional random factor to account for the repeated egg measures per female. To explore whether egg size covaries with female fecundity, we used the mean egg size per female as a response variable in a LMM. We assessed male effects on female survival using a CPHMM and on longevity with a LMM ($N = 945$), with female fecundity and weight as covariates in addition to the male age and genetic factors.

RESULTS

Senescence in females

Fecundity decreased rapidly with age in all females, and the average fecundity as well as the rate of decline depended on the mitonuclear combination (Table 1 and Supplementary Figure S1). This epistatic interaction arose from mitonuclear coadaptation: females with a coevolved mitonuclear genotype showed a higher average fecundity ($\chi^2_1 = 7.44$, $P = 0.0064$) and slower rate of decline in fecundity compared with females with a novel mitonuclear genotype (age × coevolution: $\chi^2_1 = 4.4$; $P = 0.036$, Figure 2a). Consequently, the disruption of a coadapted mitonuclear combination resulted in significantly lower lifetime fecundity (All fertile eggs: $\chi^2_1 = 5.9$; $P = 0.015$) and a marginally nonsignificant lower lifetime offspring production ($\chi^2_1 = 3.8$; $P = 0.053$, Figure 2b). We note that the lifetime fecundity difference was similar in magnitude for fertile and total number of eggs (result not shown). Female fertility declined with age (Supplementary Figure S2) but we found no significant effects of mitochondrial genetic variation or mitonuclear epistasis on this decline (Table 1). There were also no significant mitochondrial effects on actuarial ageing in females (that is, longevity and survival) or on their larval offspring survival (Supplementary Table S1). The average
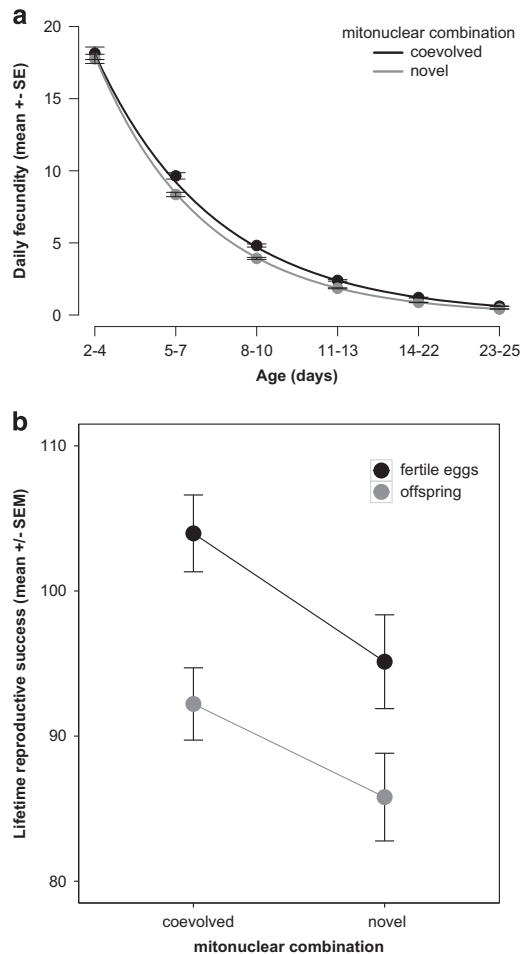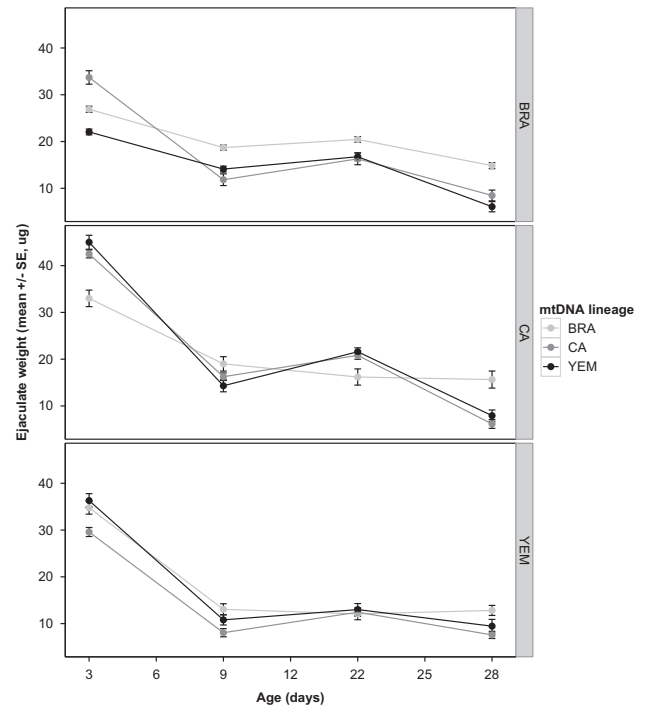
Figure 2 Daily egg-laying rate declines at a lower rate in females with a coadapted mitonuclear genotype (**a**), resulting in a higher lifetime reproductive success compared with females with a novel mitonuclear genotype (**b**). Figures show model predictions.

Figure 3 Direct mitonuclear epistatic effects on age-specific male ejaculate weight. Means represent model predictions.

longevity of females was 38 days (±0.54 s.e.) with more fecund females living longer (Supplementary Table S1).

Senescence in males

We first tested for direct mitochondrial and mitonuclear genetic effects on ageing phenotypes expressed by the males. Mitochondrial haplotype and mitonuclear combination both significantly influenced average ejaculate weight, and also its pattern of age-specific decline (Table 1 and Figure 3). As for females, there were no significant direct mitochondrial genetic effects on male longevity or survival (Supplementary Table S2). Males outlived the females from the introgression lines, with an average lifespan of 45 days (±1.49 s.e.) compared with 38 days for females.

The second aim was to test for a role for mitonuclear genotype on the indirect effects of male senescence upon reproduction and/or actuarial ageing in their mates. We found widespread evidence for both. First, male ability to stimulate female fecundity declined with age, and this decline was contingent upon both mitochondrial and nuclear lineage (Table 2 and Figures 4a and b). Here, variation in the two genomes primarily acted additively, as we found no significant mitonuclear interaction (Table 2). However, epistasis was apparent when modelled in terms of coevolutionary history: mitonuclear coadaptation had a strong impact on age-specific effects

(*coadaptation* × *nuc* × *age*: $\chi^2_6 = 23.4$; $P = 0.0007$, Figure 4b). Male ejaculate weight showed a strong positive effect on female fecundity (Table 2) but there was no interaction effect on this relationship because of the genetic factors (interactions not presented). This suggests that although ejaculate weight is an important determinant of male reproductive success and subject to mitonuclear epistasis itself, this relationship does not explain the indirect effect of male genotype on female fecundity. Second, female egg size significantly increased with male age (Table 2 and Supplementary Figure S3a), and this effect depended upon mitonuclear combination (Table 2 and Supplementary Figure S3b). When we included female fecundity into the model of egg size it removed the effect of male age (fecundity: $\chi^2_1 = 217.8$; $P < 0.00001$; male age: $\chi^2_2 = 4.3$; $P = 0.12$). This suggests that the male age effect on egg size was mediated by a maternal size number tradeoff, induced by the observed effect of male age on female fecundity. However, a mitonuclear interaction effect remained significant ($\chi^2_4 = 10.5$; $P = 0.033$). Third, there was little evidence of male ageing influence on egg fertility, but the mitonuclear genotype had a marginally nonsignificant effect on age-dependent fertility ($P = 0.053$, Table 2). However, we found some evidence for mitonuclear epistasis, as there was a significant effect of mitonuclear coadaptation on age-dependent fertility that differed between the nuclear lineages (*coevolution* × *nuc* × *age*: $\chi^2_{10} = 19.5$; $P = 0.034$). Fourth, increasing male age had a positive effect on female survival and there was also a marginally nonsignificant age × mitochondrial lineage interaction ($P = 0.055$, Table 3 and Supplementary Figure S3). This interaction was significant for female longevity ($P = 0.047$, Figure 5a), even when controlling for the strong negative effect of fecundity on female lifespan (Table 3). As for male effects on female fecundity, mitonuclear epistasis for female mates' lifespan was evident when modelled in terms of male mitonuclear coevolutionary history: there was both a significant main effect of mitonuclear coadaptation (*coevolution*: $\chi^2_1 = 4.5$; $P = 0.0336$) and an interaction with male age

Table 2 The indirect effects of male age, mitochondrial and nuclear genetic variation upon reference female fecundity, egg size and fertility

Response variable	Fecundity, n = 621					Egg size, n = 7971					Fertility, n = 954				
	Coef	s.e.	χ^2	d.f.	P	Coef	s.e.	χ^2	d.f.	P	Coef	s.e.	χ^2	d.f.	P
Fixed effects															
Male age			82.3	3	**<0.0001**			11.8	2	**0.0027**			3.2	5	0.6737
Female weight	0.03	0.02	2.2	1	0.1377	3.21	0.75	18.3	1	**<0.0001**	0.03	0.03	1.3	1	0.2525
Ejaculate weight	0.62	0.17	13.7	1	**<0.0001**	−1.50	1.24	1.4	1	0.2320	—	—	—	—	—
Mitochondrial lineage			3.2	2	0.2062			1.9	2	0.3866			0.3	2	0.8623
Nuclear lineage			0.3	2	0.8524			5.2	2	0.0750			5.9	2	0.0531
Mito × Nuc			4.3	4	0.3661			3.0	4	0.5568			5.8	4	0.2152
Age × Mito			27.2	6	**<0.0001**			17.5	4	**0.0015**			8.3	10	0.6031
Age × Nuc			39.2	6	**<0.0001**			15.9	4	**0.0031**			22.0	10	**0.0150**
Age × Mito × Nuc			15.4	12	0.2183			19.6	8	**0.0117**			31.2	20	0.0527
Random effects	Variance (σ^2)					Variance (σ^2)					Variance (σ^2)				
Line	0.00					1.51×10^{-12}					0.00				
Male ID	74.61					2.4					0.04				
Female ID	—					21.2					—				

Abbreviations: Coef, coefficient; d.f., degrees of freedom; Mito, mitochondrial lineage; Nuc, nuclear lineage. *P* < 0.05 highlighted in bold.

(*coevolution × age*: $\chi^2_5 = 13.6$; $P = 0.0183$) that also depended on the nuclear lineage (*coevolution × nuc × age*: $\chi^2_{10} = 23.1$; $P = 0.0105$, Figure 5b).

DISCUSSION

Evolutionary theories incorporating sex-specific selection predict that males and females should age differently (Bonduriansky *et al.*, 2008) and mitochondrial genetics should play a role in influencing these patterns (Frank and Hurst, 1996; Gemmell *et al.*, 2004). Here we demonstrate widespread mitochondrial genetic effects on reproductive ageing in both sexes. Our results are broadly consistent with our key predictions. First, mitochondrial genetic effects were typically contingent upon the nuclear genetic background, but the nature of this mitonuclear epistasis was sex specific. Disruption of potentially coadapted mitonuclear combinations resulted in a general increased rate of female fecundity senescence. In males, the same disruption resulted in more haphazard effects that were contingent upon the nuclear genome, male age and the trait in question. Second, mitonuclear epistasis was associated with indirect genetic effects, such that genotypic effects on reproductive senescence in males affected their female mating partners' fitness. Here, we discuss the implications of these patterns for the evolution of sex-specific reproductive strategies and the role of sexual conflict in ageing and maternal investment.

Sex-specific mitonuclear effects

Evolution of different optimal reproductive strategies in males and females should lead to sex differences in the rate of senescence (Promislow, 2003; Bonduriansky *et al.*, 2008; Maklakov and Lummaa, 2013). Empirical studies have found that sexual dimorphism in senescence is variable between species and across traits (Promislow, 1992; Chen and Maklakov, 2014; Cornwallis *et al.*, 2014). Much of this variation likely owes to interspecific differences but rates of sex-specific senescence vary even within species. This has been suggested to result from ecological conditions, differences in age-specific intensity of sexual selection (Bonduriansky *et al.*, 2008) or from intersexual genetic correlations constraining the evolution of sex-specific optima for life-history traits (Bonduriansky and Chenoweth,

2009). Asymmetric inheritance of mitochondrial genes imposes an extreme form of genetic correlation and our results demonstrate that sex-specific variation in ageing can arise from variation in mitonuclear genotypes (Maklakov and Lummaa, 2013). Although such effects have been considered for sex differences in lifespan (Camus *et al.*, 2012; Frank, 2012; Maklakov and Lummaa, 2013), to our knowledge our results are the first comprehensive report of mitochondrial and mitonuclear effects on sex-specific reproductive senescence.

The observed sex differences in mitochondrial genetic effects align well with theoretical predictions based on maternal inheritance of mitochondria (Gemmell *et al.*, 2004). Female-specific selection should thus optimise the mitochondrial function by mitonuclear coadaptation across populations. Whereas selection on the nuclear components of mitochondrial function may be quite strong in males (Gallach and Betran, 2011), this is a relatively weak engine of male-specific mitonuclear coadaptation (Wade, 2014). Our results show that females substantiate this prediction: across the nuclear genotypes, introgression of any novel mitochondrial haplotype hampered lifetime fecundity by accelerating ageing (Figure 2 and Supplementary Figure S1). This result is also in line with an increasing number of studies that show nonneutral mitochondrial effects in females (see, for example, Maklakov *et al.*, 2006; Kazancioglu and Arnqvist, 2013; Dobler *et al.*, 2014; Kurbalija Novicic *et al.*, 2014; Lovlie *et al.*, 2014). We note, however, that mitonuclear genetic variation had no significant effect on female fertility, despite the fact that fertility also showed marked ageing (Supplementary Figure S2).

Several facts suggest that optimal mitochondrial function differs between the sexes (Gemmell *et al.*, 2004; Gallach and Betran, 2011; Innocenti *et al.*, 2011), and this may help explain why male-specific mitonuclear epistasis often tends to reflect genotypic idiosyncrasies rather than general coadaptation (Maklakov and Lummaa, 2013; Dobler *et al.*, 2014). The lack of cosegregation of mitochondrial and nuclear genes in the male germ line, coupled with sex-specific selection (Rand *et al.*, 2001), can constrain male-specific mitonuclear coadaptation (Gemmell *et al.*, 2004; Bonduriansky and Chenoweth, 2009; Wade, 2014) and also contribute to the maintenance of genetic variation in male phenotypes under selection. The extent to which this is true will depend on how often mitochondrial function underlies
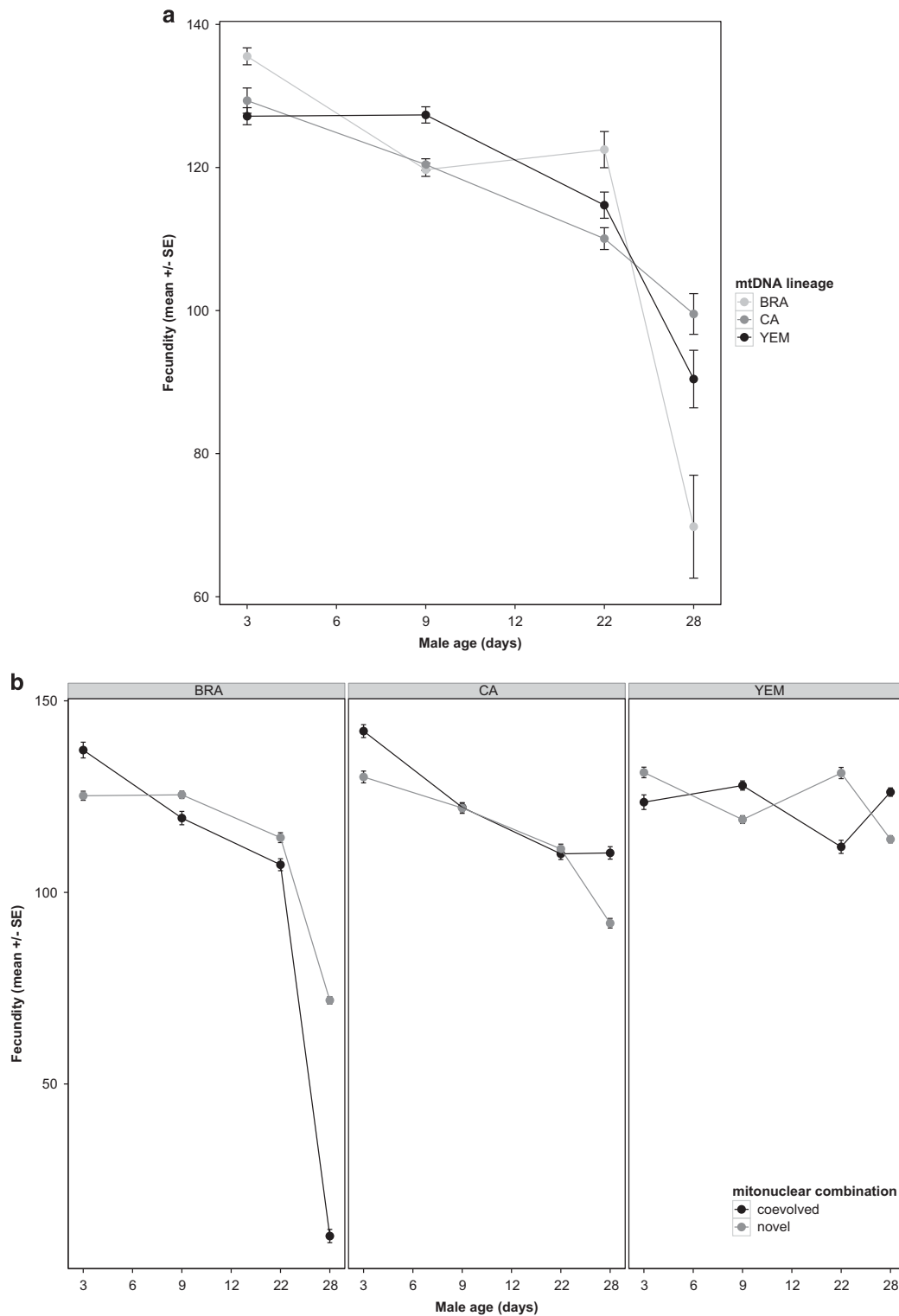
Figure 4 Indirect effects of male age and mitonuclear genotype on female reproductive traits. Male age affects female fecundity but the decline over male age differs across mtDNA haplotypes (**a**) and depends upon an interaction between mitonuclear coadaptation and the nuclear genetic background (**b**). The different nuclear lineages are shown in panels. Means represent model predictions.

male-specific phenotypes. Our results demonstrate the existence of such pathways in *C. maculatus* for traits that influence male age-specific reproductive fitness such as ejaculate weight and male fecundity stimulation (Figures 3–5).

Consistent with the above, we found that the age-specific effects of particular mitonuclear combinations are rather idiosyncratic in males. For example, the mtDNA haplotype 'BRA' showed an elevated male ability to stimulate female fecundity early in life when coexpressed

Table 3 The indirect effects of male age and mitochondrial and nuclear genetic variation on female actuarial ageing

Response variable	Female longevity, n = 945					Female survival, n = 945				
	Coef	s.e.	χ^2	d.f.	P	Coef	s.e.	χ^2	d.f.	P
Fixed effects										
Male age			6.9	5	0.2273			25.8	5	<**0.0001**
Female weight	0.36	0.38	0.9	1	0.3520	−0.0003	0.0004	1.3	1	0.2456
Lifetime fecundity	−2.07	0.42	23.9	1	<**0.0001**	0.005	0.001	47.5	1	<**0.0001**
Mitochondrial lineage			4.7	2	0.0936			3.3	2	0.1953
Nuclear lineage			1.7	2	0.4270			0.3	2	0.8810
Mito × Nuc			2.7	4	0.6078			1.4	4	0.8457
Age × Mito			18.5	10	**0.0466**			18.0	10	0.0553
Age × Nuc			15.7	10	0.1098			10.7	10	0.3836
Age × Mito × Nuc			27.7	20	0.1159			24.6	20	0.2155
Random effects	Variance (σ^2)					Variance (σ^2)				
Line	0.00					0.0042				
Male ID	1.03					0.19				

Abbreviations: Coef, coefficient; d.f., degrees of freedom; Mito, mitochondrial lineage; Nuc, nuclear lineage. P< 0.05 highlighted in bold.

with its native nuclear genome. However, this effect declined late in life but was rescued by either of the two novel nuclear genomes (Figure 4b). It is interesting to note that this rather dramatic ageing in the male performance for the 'BRA-RBA' genotype is reversed for the females of the same genotype that showed decreased rate of ageing and higher relative fecundity (Supplementary Figure S1a), implying sexually antagonistic selection (Rand *et al.*, 2001). In contrast, the 'CA' haplotype showed an elevated male fecundity stimulation in its native nuclear background both early and late in life, relative to the other haplotypes, whereas the 'YEM' nuclear lineage showed much less senescence overall. The fact that YEM nuclear lineage was capable of maintaining its performance suggests that senescence in this trait is not inevitable, but is a result of mitonuclear epistasis. Changes in ejaculate weight with age (Figure 3) do not coincide with those seen for male effects on female fecundity (Figures 4a and b), despite having an overall positive effect on female fecundity (Table 2). This suggests that age-specific male ability to stimulate female fecundity is a result of changes in ejaculate composition rather than ejaculate volume. We note that an apparent lack of mitonuclear coadaptation in males was also recently documented by Yee *et al.* (2015), who showed that the rate of decline in male mating propensity differs across mitochondrial genotypes in *Drosophila*.

Although there is now ample evidence for a role of mitochondrial malfunctioning in actuarial ageing (see, for example, Cho *et al.*, 2011; Camus *et al.*, 2012; Lee and Wei, 2012; Houtkooper *et al.*, 2013; Zhu *et al.*, 2014), our data revealed that mitochondrial genetic effects on longevity and survival were weak and nonsignificant relative to age-specific reproduction in both sexes of *C. maculatus*. This suggests that mitonuclear genetic effects on senescence are stronger for reproductive function than somatic maintenance governing lifespan, presumably reflecting the fact that reproduction is particularly sensitive to cellular malfunctioning. We have, however, previously found some evidence of mitonuclear epistasis on female lifespan under aphagous conditions (Dowling *et al.*, 2010), and this is in line with a recent report that lifespan is subject to complex mitonuclear-by-environment interactions (Zhu *et al.*, 2014).

Indirect genetic effects on female reproduction and longevity
Post-copulatory sexual selection in males has selected for a suite of male traits that influence female reproductive physiology and fitness, and many female reproductive traits may thus be subject to indirect genetic effects through males (Clark *et al.*, 1999; Edward *et al.*, 2014). To our knowledge, our results are first to demonstrate that such indirect genetic effects can involve mtDNA. We found that mitochondrial genetic variation interacted with male age to influence female fecundity and investment into eggs (that is, egg size, Supplementary Figure S3). Females mated to young males traded a high fecundity against a lower investment per egg and *vice versa* for mates of older males. In *Drosophila*, male seminal fluid proteins have well-established effects on female egg-laying rate (Chapman *et al.*, 2001; Avila *et al.*, 2011). We have recently identified >100 seminal fluid proteins in *C. maculatus*, several of which affect female fecundity (Goenaga *et al.*, 2015; Yamane *et al.*, 2015). It is likely that the effect of male age on female fitness seen in our experiments result from changes in the seminal fluid composition of male ejaculates. Because mitonuclear genotype influences the metabolic pathways that fuel biosynthesis of seminal fluid molecules (Immonen *et al.*, 2015), the indirect genetic effects seen in our experiments likely reflect differences across mitonuclear genotypes in age-related changes in seminal fluid composition of males. Interestingly, we also found evidence that female cost of reproduction was influenced by male mitochondrial genetic variation in interaction with age: male age affected female lifespan and this effect differed across mtDNA haplotypes (Figure 5a and Supplementary Figure S2), even when controlling for female reproductive effort. This lends further support for the interpretation that mitochondrial function affects ejaculate-mediated indirect genetic effects on females. Seminal fluid molecules influence female lifespan as a toxic side effect (Avila *et al.*, 2011), an effect that may change with male age because of deterioration in accessory gland function.

Taken together, our results suggest that ageing in *C. maculatus* represents both a cause and a consequence of sexual conflict. Both males and females suffer reduced reproductive output when mating with an older partner, but female ageing is further under the influence of male traits, some of which also manipulate female reproduction

a

b
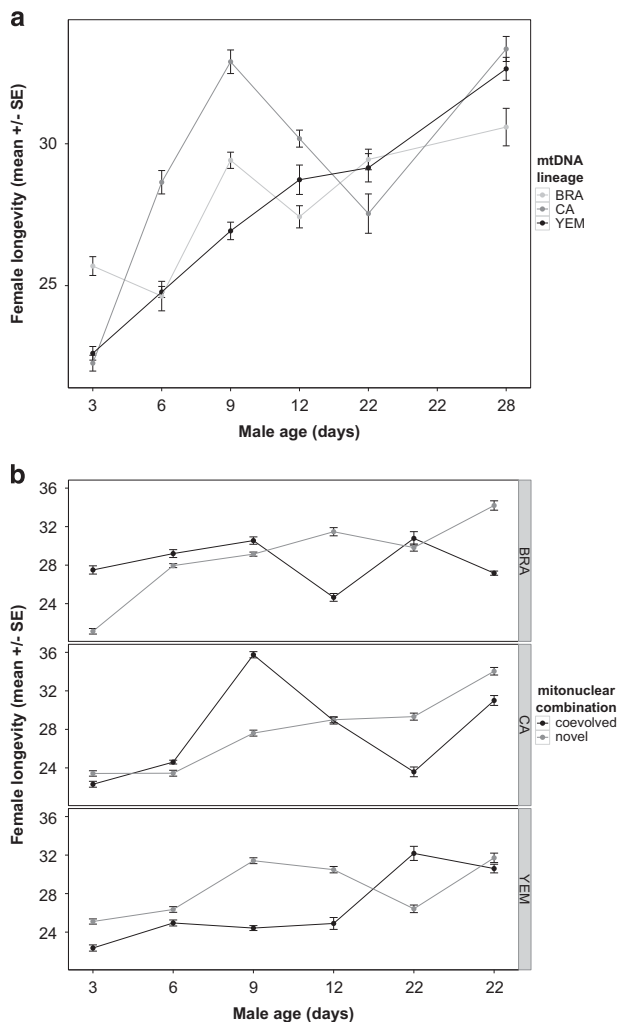
Figure 5 Female longevity increases with decreasing male age, but this general pattern also depends upon the male mtDNA haplotype (**a**) and an interaction between mitonuclear coadaptation and the nuclear genetic background (**b**). Means represent model predictions.

(fecundity and egg size investment). The mitonuclear genetic effects seen here suggest that the underlying genetic architecture of the traits involved is complex and likely to constrain male-specific adaptive evolution. This may not only depress male but also the female mating partner's reproductive success (Figure 4).

CONCLUSIONS

Our results demonstrate that mitonuclear epistatic effects are sex specific and affect reproductive, but not actuarial, ageing in this species. Mitonuclear coadaptation decelerated reproductive senescence in females, resulting in higher female fitness, whereas coexpression of mitochondrial and nuclear genomes with a shared evolutionary history had more variable and idiosyncratic effects in males. Intriguingly, we found age-specific indirect genetic effects of male mtDNA haplotype on female fecundity, egg size and longevity. This suggests an important role for mitochondrial pathways for both female fecundity and male reproduction that may be mediated through male seminal fluid substances. More generally, our results illustrate the importance of considering reproductive phenotypes in both sexes as well as the

nuclear genetic background in studies examining the role of mitochondria in ageing.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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