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Mother's curse and indirect genetic effects: Do males matter to mitochondrial genome evolution?

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Abstract

Maternal inheritance of mitochondrial DNA (mtDNA) was originally thought to prevent any response to selection on male phenotypic variation attributable to mtDNA, resulting in a male-biased mtDNA mutation load ("mother's curse"). However, the theory underpinning this claim implicitly assumes that a male's mtDNA has no effect on the fitness of females he comes into contact with. If such "mitochondrially encoded indirect genetics effects" (mtIGEs) do in fact exist, and there is relatedness between the mitochondrial genomes of interacting males and females, male mtDNA-encoded traits can undergo adaptation after all. We tested this possibility using strains of *Drosophila melanogaster* that differ in their mtDNA. Our experiments indicate that female fitness is influenced by the mtDNA carried by males that the females encounter, which could plausibly allow the mitochondrial genome to evolve via kin selection. We argue that mtIGEs are probably common, and that this might ameliorate or exacerbate mother's curse.

KEYWORDS
indirect genetic effects, interacting phenotypes, kin selection, mtDNA, mutation load, sexual conflict

1 | INTRODUCTION

In most animals and plants, cytoplasmic genetic elements such as those carried inside mitochondria, chloroplasts and endosymbiotic bacteria are, with rare exceptions, inherited only from the mother (Birky, 1995). This uniparental inheritance has been proposed to make the effects of cytoplasmic mutations on males "invisible to selection," particularly in the case of mitochondrial DNA (Beekman, Dowling, & Aanen, 2014; Frank & Hurst, 1996; Gemmell, Metcalf, & Allendorf, 2004; Hoekstra, 2000). The argument runs as follows: imagine a mutation in the mitochondrial DNA (mtDNA) that has no effect on female fitness, but which reduces the fitness of males that carry it. Even though males with the mutation leave fewer descendants (i.e., the mutation is under selection), there will be no evolutionary response since males do not transmit the mtDNA to their offspring. This hypothesis predicts that male-harming mutations will accumulate in the mitochondrial genome, either because of genetic drift coupled with ineffective selection on males (Frank & Hurst, 1996), or because some male-harming mutations have beneficial pleiotropic effects on females that cause them to be positively selected (Cosmides & Tooby, 1981; Dowling & Adrian, 2019; Smith & Connallon, 2017). The male-biased mutation load that results from uniparental inheritance is often termed "mother's curse" (Gemmell et al., 2004). Consistent with the mother's curse prediction, there is evidence that male traits have more mitochondrial genetic variance than female traits (Camus, Clancy, & Dowling, 2012; Camus & Dowling, 2018; Camus, Wolf, Morrow, & Dowling, 2015; Dowling, Tompkins, & Gemmell, 2015; Wolff, Pichaud, et al., 2016), that human

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mitochondrial disease is more prevalent in males than females (e.g., Martikainen et al., 2017; Milot et al., 2017; Wallace et al., 1988), and that many male-harming mtDNA mutations are not harmful when expressed in females (reviewed in Beekman et al., 2014; Vaught & Dowling, 2018).

However, some theoretical papers (Cosmides & Tooby, 1981; Hedrick, 2012; Unckless & Herren, 2009; Wade & Brandvain, 2009; Zhang, Guillaume, & Engelstädter, 2012) have identified specific scenarios where an evolutionary response to selection on male mtDNA is likely to occur. All of these scenarios involve kin selection (Hamilton, 1964) and "mitochondrial indirect genetic effects" (hereafter mtIGEs; Wolf, Brodie, Cheverud, Moore, & Wade, 1998), whereby the mtDNA carried by a male affects the fitness of females with which the male interacts. For example, Cosmides and Tooby (1981) and Wade and Brandvain (2009) suggested that selection might purge male-killing mtDNA mutations in species where males improve the fitness of their female relatives, for example through kin-directed altruistic behaviours or benefits from group living. A second group of models (Hedrick, 2012; Unckless & Herren, 2009; Wade & Brandvain, 2009) showed that selection will disfavour mtDNA mutations that cause males to become sterile, provided that males preferentially mate with their "mitochondrial relatives" (e.g., full sisters or maternal half-sisters). These models are all conceptually similar: their salient assumptions are that A) some male traits (e.g., behaviour or fertility) benefit the fitness of female "social partners" (e.g., group members or mates) with which the males interact, and B) male and female social partners show positive assortment with respect to their mtDNA haplotype. There is a third group of models that is also conceptually similar, except that the focus is on males harming instead of helping females. Cosmides and Tooby (1981) proposed that male-killing mtDNA mutations might be selectively favoured, provided that such mutations lessen competition on females carrying related mtDNA (e.g., the dead males' sisters). Further insightful models (Engelstädter & Charlat, 2006; Hedrick, 2012) demonstrated that selection can favour male-sterilizing cytoplasmic DNA if males mate with their relatives *less* often than expected (e.g., due to inbreeding avoidance), because this impairs the fertility of females that do not carry the male-sterilizing mutation. As before, these hypotheses can be understood in terms of kin selection (specifically "spite"; Gardner & West, 2006): the key assumptions are that males affect female fitness, and there is *negative* assortment with respect to mtDNA.

To organize and unify these hypotheses, we propose a heuristic based on Hamilton's rule ($rB - C > 0$; Hamilton, 1964), a famous inequality that describes when a social trait will be selectively favoured. If r_{mt} is the correlation in mitochondrial genotypes between interacting males and females, and B is the effect of a mitochondrially encoded male trait on female fitness, we posit that a novel mitochondrial mutation that only affects the phenotype of males will be selectively favoured when $r_{mt}B > 0$, disfavoured when $r_{mt}B < 0$, and neutral when $r_{mt}B = 0$. We can omit the cost term C because mitochondrial mutations carried by males always have a direct fitness of zero under uniparental inheritance, and so $C = 0$. The simple expression $r_{mt}B > 0$ unifies the models above: when r_{mt} is positive (negative), meaning that interacting males and females are more (less) likely to carry the same mitochondrial mutation as two randomly chosen individuals, selection favours mitochondrial mutations that increase (decrease) the fitness of female social partners, all else equal. If the mitochondrial mutation has no indirect genetic effect on female fitness ($B = 0$), or if interacting males and females assort at random with respect to their mtDNA ($r_{mt} = 0$), then the mutation will be selectively neutral; this recapitulates the findings of models such as Frank and Hurst (1996), which implicitly assumed that interactions are random and/or that mtIGEs do not exist.

To our knowledge, no empirical study has tested whether kin selection of this kind actually occurs. Immonen, Collet, Goenaga, and Arnqvist (2016) conducted what appears to be the first and only measurement of mitochondrial indirect genetic effects, and found that male mtDNA affected the fecundity, egg size and egg hatching rate of the male's mates in *Callosobruchus maculatus* beetles (though the paper did not mention the implications of this result for mother's curse). Additionally, studies of *Drosophila melanogaster* fruit flies have found that mitochondrial genetic variance affects several male phenotypes, many of which seem likely to affect the fitness of the male's female social partners. These traits include metabolic rate (Arnqvist et al., 2010; Đorđević et al., 2017) and body size (Dobler, Rogell, Budar, & Dowling, 2014), which potentially influence how males influence female fitness during behavioural interactions (e.g., large, highly active males might be more harmful to females; Pitnick & García-González, 2002). Additionally, variance in mtDNA strongly affects male sperm function (Dowling, Nowostawski, & Arnqvist, 2007; Patel et al., 2016), and multiple mtDNA mutations can cause total male sterility when paired with particular nuclear genotypes (Clancy, Hime, & Shirras, 2011; Dowling et al., 2015; Xu, DeLuca, & O'Farrell, 2008).

Here, we experimentally test one of the two essential conditions required for male mtDNA evolution: that the mtDNA haplotype carried by male *D. melanogaster* affects the fitness of the females they encounter. In other words, we test whether the B term in our modified Hamilton's rule differs from zero. *Drosophila* males can have strong effects on female fitness through both behavioural interactions (e.g., males continually harass females and attempt to mate; Le Page et al., 2017; Partridge & Fowler, 1990) and mating (e.g., females need functional sperm, and male seminal fluid profoundly affects female physiology; Chapman, 2008; Perry, Sirot, & Wigby, 2013). Therefore, our first experiment tests for a causal effect of male mtDNA on the fitness of co-habiting females, by experimentally manipulating males' mtDNA haplotypes while holding constant other variables. We also varied the presence/absence of males, to test whether the effect of male presence depends upon what mtDNA the males carry. We also conducted a second, similarly designed experiment to measure the direct effect of mtDNA on the fitness of females themselves, allowing us to compare the magnitude of the indirect and direct genetic effects of mtDNA on female fitness.

2 | METHODS

2.1 | Drosophila cultures

2.1.1 | Mitochondrial strains

We used five strains of *D. melanogaster* sourced from a mitochondrial reference panel (Camus et al., 2012; Wolff, Camus, Clancy, & Dowling, 2016). Each strain carries a distinct mtDNA haplotype originally sourced from a diverse global origin; the strains were established by Clancy (2008) and are named after the geographic locations where their mtDNA originated. All strains have the same isogenic nuclear genome: the commonly used genotype w^{1118} (Bloomington stock number: 5,905). The strains were propagated by backcrossing virgin females from each strain to males from a single stock of w^{1118} for over 100 generations prior to use in our experiments. Furthermore, the w^{1118} stock used to source the males for these backcrosses is maintained by mating one full-sibling pair per generation. This ensures the w^{1118} stock is virtually homozygous at all loci in the nuclear genome, and prevents the nuclear genomes of each of the mitochondrial strains from diverging over time. To ensure that cytoplasmic genetic differences were limited to the mitochondrial genome, the strains were treated with tetracycline to remove any infections by intracellular bacteria, such as *Wolbachia*, dozens of generations before this study. One of the mitochondrial strains we examined (Brownsville) is known to cause total male sterility when paired with the w^{1118} nuclear background; the other four strains are fertile (Camus & Dowling, 2018; Clancy et al., 2011).

2.1.2 | DGRP lines

To test for male mtDNA-encoded phenotypic effects on females during Experiment 1, we utilized two inbred, wild-type strains from the *Drosophila* Genetic Reference Panel (DGRP; Mackay et al., 2012). Like our mitochondrial strains, DGRP strains have a nuclear genome that is >99% homozygous, which should help minimize extraneous variation in female fitness; we arbitrarily selected the DGRP strains from among the ~200 available by picking stocks that were easy to culture in our laboratory. All the females in Experiment 1 came from the DGRP-517 strain (Bloomington: 25,197), whereas the nonmitochondrial strain males came from DGRP-352 (Bloomington: 28,177). Finally, in Experiment 2, we exposed mitochondrial strain females to males from DGRP-324 (Bloomington stock no. 25,182).

All flies were cultured on standard cornmeal food medium (adapted from Brent & Oster, 1974) at 25°C, under natural light. To reduce extraneous phenotypic variation, all flies used in the experiments were reared at a standardized density by adding 100 first-instar larvae to each food vial. Each mitochondrial strain was reared in several different vials. We collected recently eclosed (<4 hr) virgin flies under light CO_2 anaesthesia and housed them in single-sex groups to mature for 3–4 days, at which point they were used in the experiments. Experiments 1 and 2 were run concurrently and were each partitioned into seven blocks of roughly equal size; each mitochondrial strain was equally represented within each block. Experiment 1 measured the fitness of 217 DGRP-517 females, whereas Experiment 2 measured 169 mitochondrial strain females.

2.1.3 | Experiment 1: Effects of male mtDNA on female fitness

We tested for effects of male mtDNA on female fitness by measuring female offspring production, in the presence of different mitochondrial strain males. To begin the experiment, we placed pairs of virgin flies (a DGRP-517 female and a DGRP-352 male) into each of several food vials, and selected pairs that were observed mating for use in the next phase of the experiment. The purpose of this initial mating was to ensure every female had mated with a standardized male genotype at the commencement of the experiment, removing one possible source of variation in female offspring production. Additionally, one of our mitochondrial strains, Brownsville, has sterile males (in the w^{1118} nuclear background), and so the fitness of females housed with Brownsville males would always be zero had we not provided this initial mating plus continued access to a DGRP-352 male. We expect that females housed with Brownsville males would not be sperm limited in the first few days of the experiment since they had just mated, but might have become sperm limited in the latter days.

Following mating, we randomly and evenly divided the mated pairs between two treatments, termed "high male exposure" and "low male exposure." In the high male exposure treatment, we introduced three virgin males—all from one of the mitochondrial strains—to each vial. The original DGRP-352 male was left in the vial and accompanied the female throughout the experiment (i.e., four males and one female per vial). In the low male exposure treatment, no additional males were added, so the vial contained only the female and her DGRP-352 mate. In both treatments, we pushed down the cotton plug of each vial to restrict the flies to a volume of around $9cm^3$, making behavioural interactions more frequent (Figure 1).

On day 3 (72 ± 2 hr after the initial set up), we transferred the females from the high male exposure treatment to the low male exposure treatment, and *vice versa*. This was accomplished by aspirating the female (with her original DGRP-352 mate) into a fresh vial and adding either zero (low male exposure) or 3 (high male exposure) six- to seven-day-old mitochondrial strain males. We repeated this procedure on days six and nine, such that females spent three days in each of four vials as they aged—two low male exposure vials, and two high male exposure vials (Figure 1). Within experimental blocks, we used the same pool of males (within each mitochondrial strain) throughout, such that the males aged alongside the females, but we rotated them between females at each treatment change, so that females never spent more than three days with the same individual mitochondrial strain males (to minimize any sexual familiarity effects; see Tan et al., 2013). Dead males were replaced with males of the same age and genotype at the time of female vial transition; these males came from a same-aged stock reared alongside the main experiment at the same

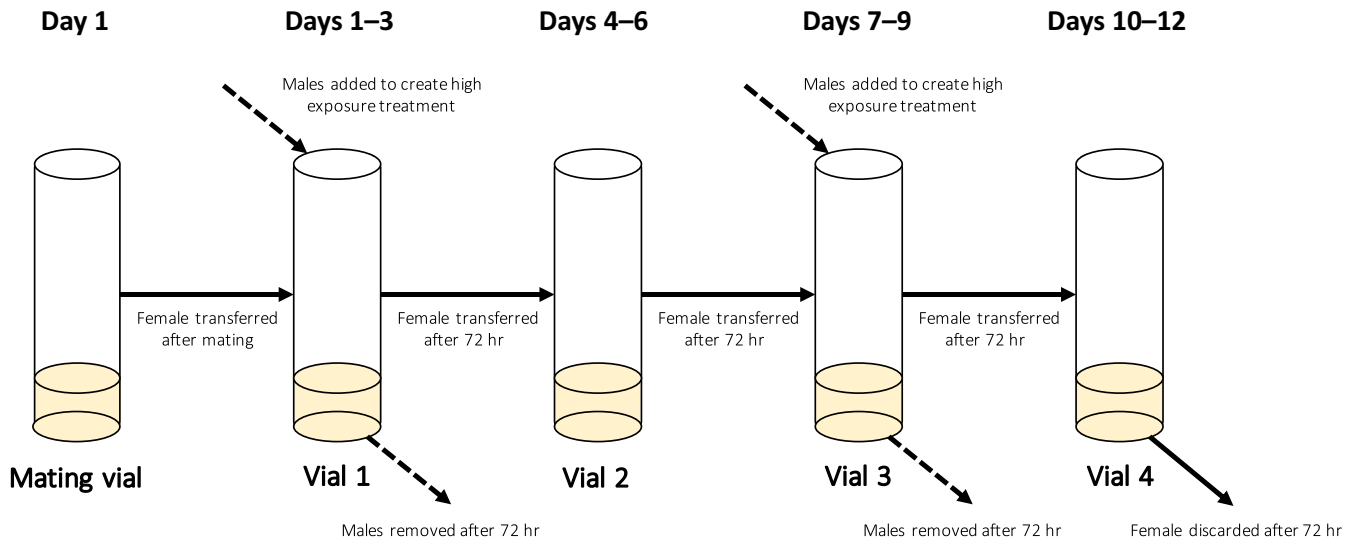

FIGURE 1 Schematic illustration of the experimental design employed in Experiments 1 and 2. Black arrows represent either the transfer of a DGRP-517 female with her initial DGRP-352 mate into a new vial in Experiment 1, or a female from one of the five mitochondrial strains into a new vial in Experiment 2. Dashed lines indicate the transfer of three males into or out of vials. In Experiment 1, the male triad belonged to one of the five mitochondrial strains, whereas in Experiment 2, males from the 324 DGRP line were used. The illustration shows an example of the "exposed first" treatment, as the female is exposed to the high male density treatment in her first and third vials. In the "exposed second" treatment, females were exposed to a high male density in their second the fourth vials

4:1 male:female ratio as in the high male exposure treatment. We monitored each vial daily until day 12 or until the female died. To assess female fitness, we counted the number of offspring produced from each vial. This was accomplished by keeping all vials for a further 13 days after removal of the female, at which time they were frozen; we later counted the number of male and female adult progeny.

2.1.4 | Experiment 2: Direct effects of female mtDNA on fitness

To assess the direct effect of mtDNA haplotype on female fitness in a similar experimental context, individual three- to four-day-old virgin females from each of the five strains were mated with age-matched virgin DGRP-352 males. Following copulation, the male was discarded, and each female was randomly allocated to either a high male exposure treatment or a no-male treatment (Figure 1). Females in the high male exposure treatment were housed with three DGRP-324 males, whereas females in the no-male treatment were housed alone (Figure 1). As with Experiment 1, females were alternated between the two treatments every three days over a 12-day period or until they died; the volume in the vial was reduced to *c*. 9 cm^3; females were never exposed to the same males twice, and dead males were replaced daily with same-aged males reared at the same-sex ratio as in the high male exposure treatment. The total number of offspring produced in the four vials from each female was assessed as for Experiment 1.

2.2 | Statistical analysis

We analysed the results of both experiments using Bayesian multivariate generalized linear mixed models implemented in the *brms* package for R, which interfaces with the Stan statistical programming language (Bürkner, 2017). All models of offspring production assumed a zero-inflated negative binomial distribution; this distribution was chosen because the response variable is overdispersed count data containing an excess of zeros. As the response variable, we used the number of offspring eclosing from each of the focal female's four vials, with the four offspring counts forming a 4-component multivariate observation. This formulation allowed each of the four offspring counts to have its own intercept (allowing productivity to change nonlinearly as females aged), and allowed for correlations between vials belonging to the same female. We chose to use multivariate models (rather than a univariate model with repeated measures in each vial) for the practical reason that it makes it easier to place a different prior on data from vial 1, which was necessary because of the design of Experiment 1. In Experiment 1, half of the females never encountered mitochondrial strain males until their second vial, and so we have a strong expectation that the effect of male mtDNA should be zero in vial 1 for these females. We therefore imposed a prior that constrained the effect of male mitochondrial strain on offspring production in vial 1 to be zero for females in the "Exposed second" treatment. In a pilot analysis, we found that analysing the data using a more traditional univariate model, with Vial as a fixed factor and *brms*' default (weak) priors, gave qualitatively identical results despite the incorrect prior. With the exception of the special prior just discussed, we used *brms*' default weak priors. Lastly, all models of Experiment 1 dealt with missing values (which resulted from the accidental loss of 16 vials in Experiment 1 and three vials in Experiment 2 before they were counted) by imputing them using the R package *mice*, and pooling the results of ten models run on

10 sets of imputed data (via the brm_multiple function in *brms*); this imputation prevented the need to discard data for females for which <4 vials were counted.

For both experiments, the "full model" contained the predictors *Mitochondrial strain*, a 5-level factor describing the mtDNA haplotype carried by the focal males or females, *Order of exposure*, a 2-level factor identifying whether the female was housed with multiple males in vials 1 and 3 (the "Exposed first" treatment), or in vials 2 and 4 ("Exposed second"), and their 2-way interaction; we also included experimental block as a random effect. To evaluate competing causal hypotheses, we fit these full models and all their component models (see Tables 1 and 2), and ranked them by their posterior model probabilities (estimated using bridge sampling via the post_prob function in brms; Gronau, Wagenmakers, Heck, & Matzke, 2019). Posterior model probabilities can be interpreted as the probability that each model is the best fitting one in the set under comparison.

We also used the full model to calculate the posterior distribution for the treatment group means, as well as various informative differences between treatment group means. For example, we computed the posterior for the pairwise differences in total offspring production between females housed with the various mitochondrial strain males in Experiment 1. We also estimated how much these pairwise differences differed between the "Exposed first" and "Exposed second" treatments. This exercise serves a similar purpose to post hoc tests or planned contrasts. Following convention, we interpret differences for which the 95% credible intervals exclude zero as noteworthy. Note that these pairwise comparisons do not constitute multiple independent tests: all of the information used to calculate each comparison is contained in the model's posterior, and thus, it is not necessary to apply false discovery rate correction.

In Experiment 2 (where 34/169 females died during the experiment), we ran a survival analysis to test for mtDNA effects on female mortality up to day 12. The response variable was the vial in which the female died, and we fit a Weibull-distributed mixed model with right-censoring implemented in *brms*. We fitted *Mitochondrial strain*, *Order of exposure* and their interaction as fixed effects, with experimental block as a random effect. Very few females died during Experiment 1 (8 out of 224), so we did not perform a survival analysis.

Finally, we tested whether mtDNA haplotype affected offspring sex ratio, using a separate model for each experiment. This was accomplished by fitting a binomial mixed model with block and female ID as random effects, and mtDNA haplotype as a fixed effect. We compared posterior model probabilities for models with and without the mtDNA haplotype effect to test for effects on the sex ratio.

TABLE 1 Model selection table for Experiment 1

Fixed effects	Posterior model probability
Male mtDNA × Order of exposure	0.78
Male mtDNA + Order of exposure	0.18
Male mtDNA	0.04
Order of exposure	0.01
Intercept-only	0.00

Note: Five multivariate mixed models were compared, which differed only in their fixed effects. The most complex model had the highest posterior model probability, but a simpler model lacking the 2-way interaction was also plausible given the data. The table suggests that offspring production was affected by male mitochondrial strain, and that this effect might differ between "Order of exposure" treatments. We used a flat prior, meaning that all models were considered equally likely.

3 | RESULTS

3.1 | Experiment 1: Effects of male mtDNA on female fitness

The mtDNA carried by males significantly predicted the number of progeny produced by females that they interacted with. Figure 2 shows the values of the group means predicted by the full model, Table 1 compares the fit of different models to the data, Table 2 and Figure 4 summarize the pairwise differences in offspring production between mitochondrial strains, and Tables S1–S6 give complete model results, group means and summary statistics.

The three models that included male mitochondrial strain as a predictor topped the list of models and had a combined posterior model probability of >99% (Table 1), demonstrating that variation in the mtDNA haplotype harboured by males influences how these males affect female offspring production. Specifically, the posterior estimate of total progeny production (i.e., the sum of the estimates in vials 1–4) was higher for females living with Dahomey or Israel males, relative to Barcelona, Brownsville or Sweden males—at least in the "Exposed first" treatment (Figure 4; Table 2). When exposed second, only the Brownsville versus Dahomey or Israel comparisons remained statistically significant; Brownsville was also significantly lower than Sweden in the "Exposed second" treatment (Figure 4; Table 2).

The best supported model included the interaction between male mitochondrial strain and *Order of exposure*, although models lacking this interaction were also plausible (Table 1; posterior probability = 22%). To investigate further, we estimated the difference in mean progeny production of females that were initially exposed to males in their first versus second vial, separately for each male mtDNA haplotype group, and then compared the size of this difference for each pair of mitochondrial strains. Only one difference was statistically significant: females paired with Dahomey males were more strongly affected by the *Order of exposure* treatment than were females paired with Sweden males (Table S4).

Lastly, we found minor differences in offspring sex ratio between mitochondrial strains (Figure S1). However, the between-strain variation was not significantly greater than expected by chance (Table S6).

TABLE 2 Posterior estimates of the differences in mean offspring production for each possible pair of male haplotypes in Experiment 1, summed across the four vials and split by "Order of exposure" treatment

Haplotype 1	Haplotype 2	Order of exposure	Difference in offspring production	*SE*	Relative difference
Brownsville	Barcelona	Exposed first	−5.05 (−18.47 to 8.47)	6.85	0.07 (0 to 0.2)
Dahomey	Barcelona	Exposed first	15.79 (1.62 to 30.58)	7.36	0.13 (0.02 to 0.24)*
Dahomey	Brownsville	Exposed first	20.84 (6.19 to 36.11)	7.63	0.17 (0.06 to 0.28)*
Dahomey	Israel	Exposed first	1.35 (−13.81 to 16.56)	7.72	0.05 (0 to 0.15)
Dahomey	Sweden	Exposed first	19.95 (5.89 to 34.74)	7.34	0.17 (0.05 to 0.27)*
Israel	Barcelona	Exposed first	14.44 (0.42 to 29.01)	7.26	0.12 (0.01 to 0.23*
Israel	Brownsville	Exposed first	19.49 (4.95 to 34.5)	7.52	0.16 (0.05 to 0.27)*
Sweden	Barcelona	Exposed first	−4.16 (−17.22 to 8.63)	6.56	0.06 (0 to 0.19)
Sweden	Brownsville	Exposed first	0.88 (−12.54 to 14.14)	6.78	0.05 (0 to 0.15)
Sweden	Israel	Exposed first	−18.6 (−33.14 to −4.77)	7.24	0.19 (0.05 to 0.35)*
Brownsville	Barcelona	Exposed second	−7.3 (−18.32 to 3.38)	5.52	0.09 (0 to 0.23)
Dahomey	Barcelona	Exposed second	4.98 (−6.31 to 16.39)	5.77	0.06 (0 to 0.16)
Dahomey	Brownsville	Exposed second	12.28 (1.78 to 23.24)	5.46	0.13 (0.02 to 0.23)*
Dahomey	Israel	Exposed second	−5.09 (−16.77 to 6.51)	5.91	0.07 (0 to 0.18)
Dahomey	Sweden	Exposed second	−1.77 (−13.16 to 9.52)	5.78	0.05 (0 to 0.15)
Israel	Barcelona	Exposed second	10.07 (−1.47 to 21.76)	5.89	0.1 (0.01 to 0.2)
Israel	Brownsville	Exposed second	17.37 (6.43 to 28.96)	5.73	0.17 (0.07 to 0.27)*
Sweden	Barcelona	Exposed second	6.75 (−4.71 to 18.41)	5.87	0.08 (0 to 0.18)
Sweden	Brownsville	Exposed second	14.05 (3.34 to 25.35)	5.61	0.14 (0.04 to 0.24)*
Sweden	Israel	Exposed second	−3.32 (−15.08 to 8.47)	5.98	0.06 (0 to 0.16)

Note: Males from the Dahomey, Israel and Sweden haplotypes were associated with higher offspring production than the Brownsville haplotype, and there were also differences between Dahomey and Sweden, Dahomey and Barcelona, and Israel and Sweden (particularly in vial 2; see Figures 2 and 4). Asterisks mark statistically significant differences. The numbers in parentheses are 95% credible intervals. The "Relative difference" column gives the absolute difference in means divided by the mean for haplotype 1

3.2 | Experiment 2: Effects of female mtDNA on fitness under high male density

The number of progeny produced by females carrying different mtDNA haplotypes varied, though posterior model probabilities suggest that the results were equivocal (Figures 3 and 4, Table 3). Full model results, group means and summary statistics pertaining to Experiment 2 are given in Tables S7-S13.

The posterior model probabilities indicate that a female's mtDNA had little effect on the number of offspring produced: mtDNA genotype did not appear in either of the top two models (Table 3). Despite the low amount of variation explained by female mtDNA genotype, there was evidence that Barcelona females were less productive (particularly relative to Brownsville, Israel, and Sweden), at least in the treatment group that encountered males in their second vial (Figures 3 and 4; Table 4). Dahomey females were also somewhat less fit.

The effect of the *Order of exposure* treatment was significantly stronger for Barcelona females than for all other haplotypes in at least one age category (Figure 4; Table S9), implying that a female's mtDNA influences how much her fitness is affected by interacting with males. However, this conclusion was (at best) weakly supported: the best ranked model that included the mtDNA x *Order of exposure* 2-way interaction had a posterior model probability <1% (Table 3).

A mixed effects survival analysis suggested that neither female mtDNA, *Order of exposure* treatment nor their interaction affected the likelihood of surviving the 12-day experiment (Tables S11–S12). Lastly, the offspring sex ratio was broadly similar between haplotypes (Figure S1), and this variation was not significantly greater than expected by chance (Table S13).

4 | DISCUSSION

Our experiment was predicated on the assertion that the male-specific effects of maternally inherited genes (such as mtDNA) can respond to selection, provided that two conditions are met. Firstly, the mtDNA of a male must affect the fitness of females he interacts with; that is, there must be a mitochondrial indirect genetic effect (mtIGE). Secondly, males and females must interact nonrandomly with respect to their mtDNA (i.e., there must be "mitochondrial relatedness" between interacting individuals; $r_{mt} \neq 0$). Experiment 1 provides experimental evidence that the first condition can be met in *D. melanogaster*: manipulating the mitochondrial genotype of interacting

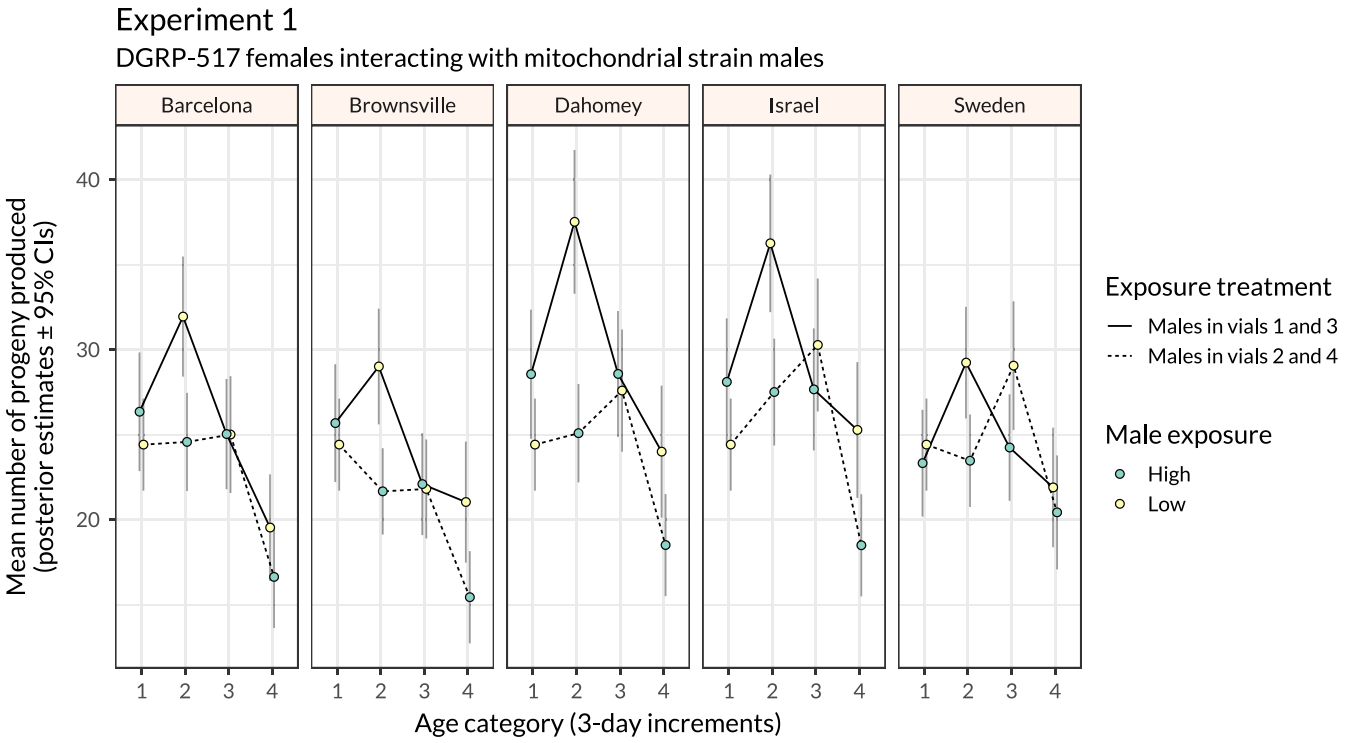

FIGURE 2 The mean number of progeny produced by females in Experiment 1, for each combination of predictor variables. The coloured points (green = high male exposure, yellow = low male exposure) show the posterior estimate of the mean, using the model shown in Table S1, and the error bars show 95% credible intervals. Dashed lines denote females exposed to the high male density treatment in their first and third vials; solid lines denote females exposed to the high male density treatment in their second and fourth vials

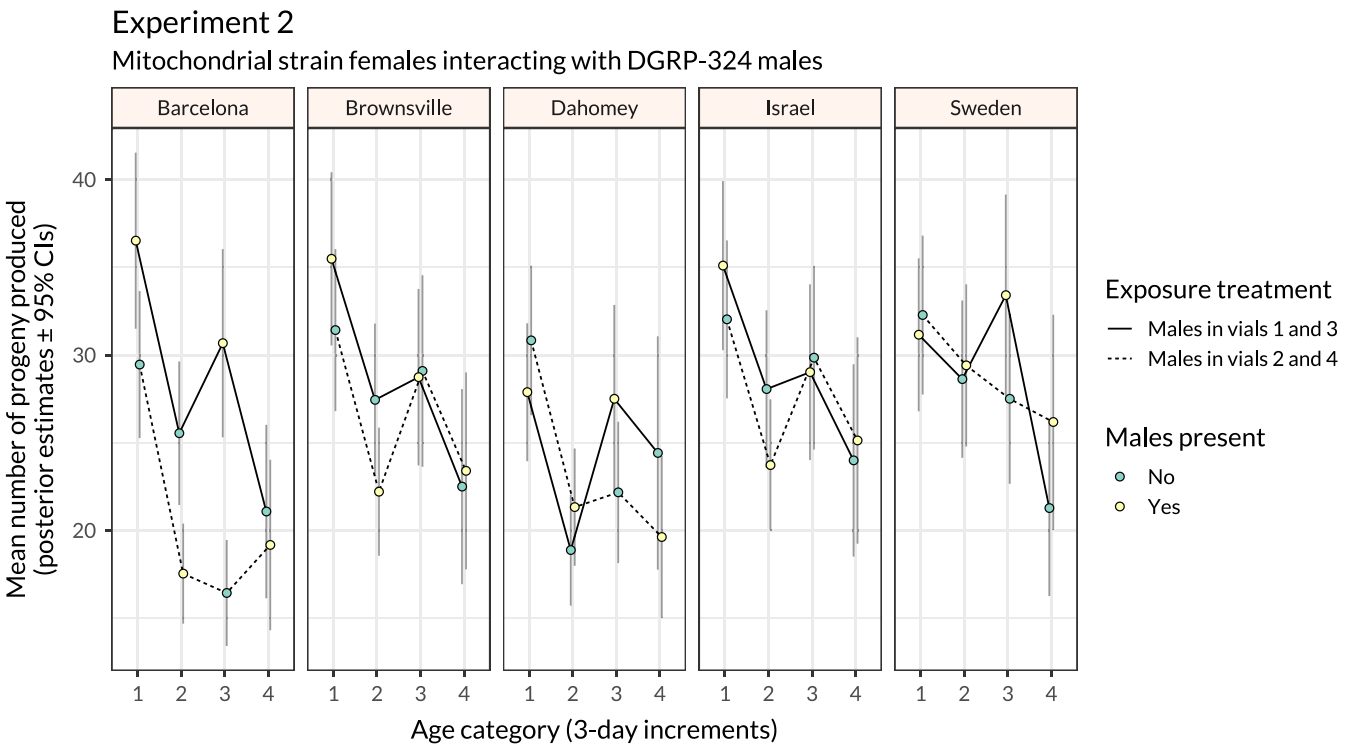

FIGURE 3 The mean number of progeny produced by mitochondrial strain females in Experiment 2, for each combination of predictor variables. The coloured points (green = males present, yellow = males absent) show the posterior estimate of the mean, using the model shown in Table S6, and the error bars show 95% credible intervals

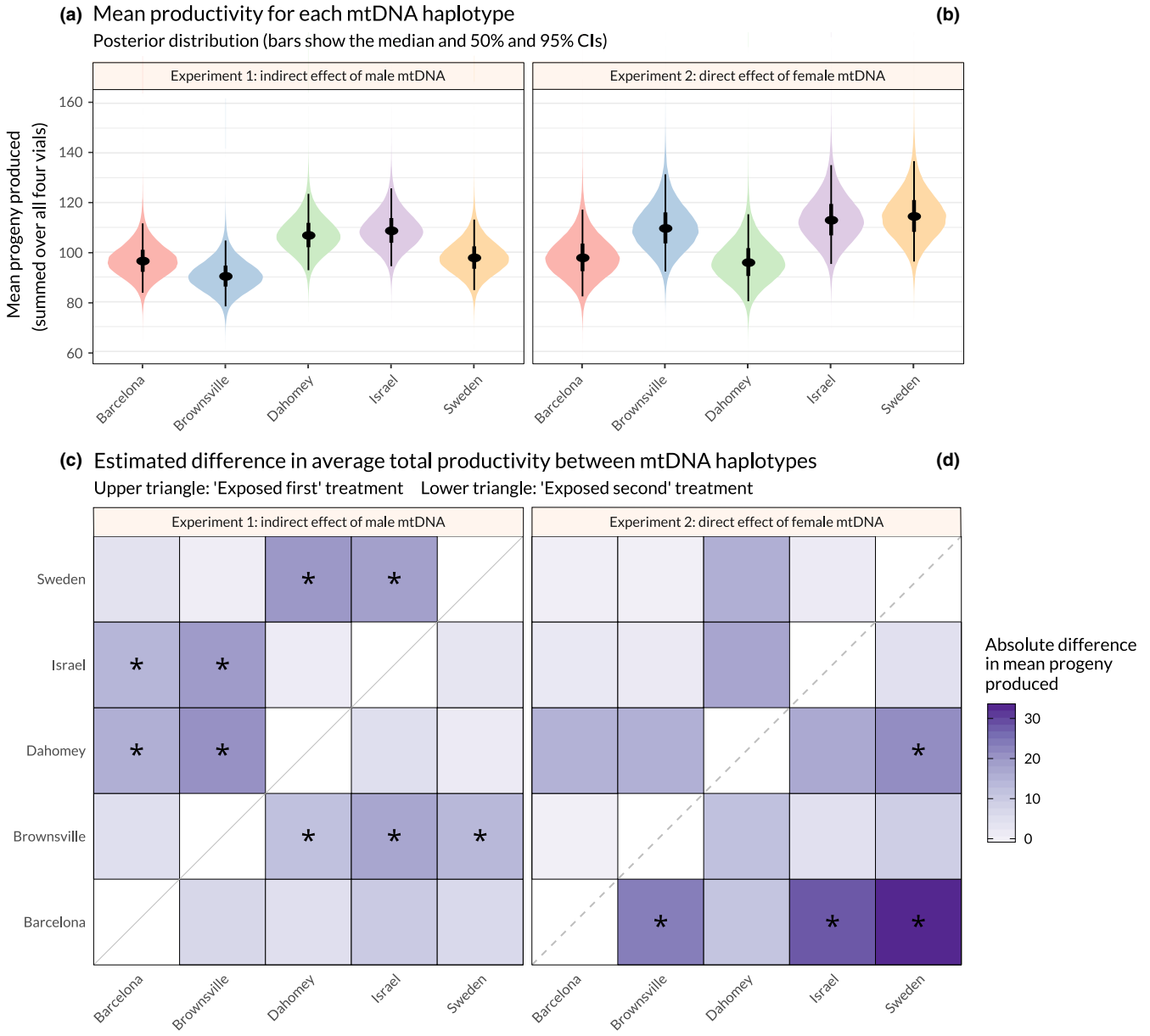

FIGURE 4 The effects of mtDNA on female fitness. (a) The estimated indirect effects of mitochondrial haplotypes in males on female productivity. (b) The estimated direct effects of mitochondrial haplotypes on female productivity. (a–b) Black points represent the median of the posterior estimate of total offspring production over the entire experiment, innermost bars show 50% credible intervals, outermost bars show 95% credible intervals, and the coloured areas show the whole posterior (calculated from the full models of each experiment). **c** summarized differences in mean female productivity for pairs of male mtDNA haplotypes during Experiment 1, split between the "Exposed first" (upper triangles) and "Exposed second" (lower triangles) treatments. (d) summarized differences in mean female productivity for pairs of female mtDNA haplotypes during Experiment 2, split between the "Exposed first" (upper triangles) and "Exposed second" (lower triangles) treatments. (c–d) The graphs show posterior estimates calculated from the full models of each experiment; asterisks mark comparisons for which the 95% credible intervals associated with this difference did not overlap zero

males altered the fitness of co-habiting females. Our study does not prove that such a similar mtIGE is also present in wild populations under field conditions, nor did we test whether $r_{mt} \neq 0$ in the wild. Therefore, it would be premature to conclude that a response to selection on male mtDNA-encoded phenotypes occurs in this species, in nature. Nonetheless, it seems plausible that similar mtIGEs exist in the wild, particularly for mtDNA haplotypes with strong effects on fertility, such as the Brownsville haplotype. Additionally, it is well-documented that *D. melanogaster* males are capable of kin discrimination in the context of mating (Carazo, Tan, Allen, Wigby, & Pizzari, 2014; Le Page et al., 2017), and there is evidence to suggest that mating pairs assort by relatedness in some populations (Loyau, Cornuau, Clobert, Danchin, & Bilde, 2012; Robinson, Kennington, & Simmons, 2012a, 2012b).

In Experiment 1, females housed with males carrying the Brownsville haplotype had reduced fitness. Though we ensured

TABLE 3 Model selection table for Experiment 2

Fixed effects	Posterior model probability
Intercept-only	0.83
Order of exposure	0.17
Female mtDNA × Order of exposure	0.00
Female mtDNA + Order of exposure	0.00
Female mtDNA	0.00

Note: Five multivariate mixed models were compared, which differed only in their fixed effects. The intercept-only model had the highest posterior model probability, though a model containing "Order of exposure" was also quite probable. Models containing "Female mtDNA" were ranked low, suggesting that the mitochondrial strain of the females had little or no effect on offspring production. We used a flat prior, meaning that all models were considered equally likely.

that all females mated with at least one fertile male at the start of both experiments, the decrease in offspring production caused by Brownsville males might well be explained by the previously documented infertility of these males (Camus & Dowling, 2018; Clancy et al., 2011). *Drosophila* females primarily use sperm from their most recent mate (Gromko, Gilbert, & Richmond, 1984; Snook & Hosken, 2004), so females that re-mated to a Brownsville male might have impaired fertility. The Brownsville haplotype contains a point mutation in the mitochondrial gene *Cytochrome B* which appears to cause total male sterility when paired with the w^{1118} nuclear genome used in our experiments (Camus et al., 2015; Clancy et al., 2011). Moreover, the Barcelona mtDNA haplotype had the second lowest male fertility after the Brownsville haplotype in a previous study (Camus & Dowling, 2018) and was associated with the second worst offspring production in Experiment 1; this again suggests that mitochondrially encoded variation in male fertility underlies some, potentially all, of the mtIGEs we observed.

Our data do not exclude the possibility that male traits other than fertility vary between mitochondrial strains. Future studies could examine whether mtDNA influences the behaviour of males towards females, for example by altering the frequency or severity of female-harming male behaviours such as persistent courtship (Partridge & Fowler, 1990). Nuclear genetic variance in male-induced harm has been demonstrated for *D. melanogaster*, and this nuclear indirect genetic effect explains a significant proportion of the variance in female egg production (Filice & Long, 2016). Similar variance stemming from the mitochondrial genome is plausible given the energetically demanding courtship behaviour of *D. melanogaster* males, and the close association between mtDNA and respiration (Dowling & Adrian, 2019; Koch & Hill, 2018; Liu, Fiskum, & Schubert, 2002).

In Experiment 2, we found some evidence that females carrying different mitochondrial haplotypes varied in their reproductive output, consistent with the results of similar experiments in insects (Camus & Dowling, 2018; Dowling, Maklakov, Friberg, & Hailer, 2009; Immonen et al., 2016). Females carrying the Dahomey and Barcelona haplotypes had reduced offspring production compared to Brownsville, Israel and Sweden. Surprisingly, Barcelona strain females showed a uniquely strong response to the *Order of exposure* treatment relative to the other four haplotypes, suggesting elevated sensitivity to the presence/absence of male within particular age classes. One possible explanation for this result is that females interacting with males need to undertake energy-intensive behaviours such as decampment or kicking in response to excessive courtship (Blanckenhorn et al., 2002; Jormalainen, Merilaita, & Riihimäki, 2001; Rowe, 1994). Variation in these behaviours might be partly explained by variation in mtDNA, given the relationship between mtDNA and respiration (Kurbalija Novičić et al., 2015; Wolff, Pichaud, et al., 2016).

Though experimental evidence that male mtDNA has indirect fitness effects on females is currently scarce (present study; Immonen et al., 2016), we believe it is very likely that selection on male mtDNA has evolutionary consequences in at least some species with strict maternal inheritance of mtDNA. This is because variation in mtDNA affects so many male traits across a variety of species (Camus et al., 2012; Camus & Dowling, 2018; Dobler et al., 2014; Immonen et al., 2016; Innocenti, Morrow, & Dowling, 2011; Milot et al., 2017; Mossman, Biancani, Zhu, & Rand, 2016; Nakada et al., 2006; Smith, Turbill, & Suchentrunk, 2010; Trifunovic et al., 2004; Wolff, Pichaud, et al., 2016; Yee, Sutton, & Dowling, 2013), and because kin-directed interactions are so common in nature. For example, some species have obligate sib-sib inbreeding, including fig wasps (where siblings mate inside a sealed fig; Herre, West, Cook, Compton, & Kjellberg, 1997) and some haplodiploid mites (where mothers mate with their asexually produced haploid sons if males are not available; McCulloch & Owen, 2012); mtDNA that caused male infertility would be selected out in these species. Interactions between males and females with negative mitochondrial relatedness are also likely to be common because of inbreeding avoidance (Szulkin, Stopher, Pemberton, & Reid, 2013) and sex-biased dispersal (Li & Kokko, 2019). Moreover, it is uncontroversial that kin-selected, male-specific adaptations can evolve in other maternally transmitted genetic elements, such as the endosymbiotic bacteria *Wolbachia* and *Spiroplasma*. For example, some strains of *Wolbachia* and *Spiroplasma* kill males that inherit them, which removes these males as competitors and thereby confers indirect benefits to related symbionts inside the dead males' female relatives (Hurst, 1991). Also, some *Wolbachia* strains induce "cytoplasmic incompatibility" (Hoffmann & Turelli, 1988; Stevens & Wade, 1990; Stouthamer, Breeuwer, & Hurst, 1999; Yen & Barr, 1971), in which the sperm of *Wolbachia*-infected males are modified in such a way that they inactivate the eggs of uninfected females upon fertilization, which increases the relative fitness of *Wolbachia*-infected females (whose eggs are protected from inactivation).

In the Introduction, we noted that although mtIGEs allow adaptation of male phenotypes encoded by mtDNA, the strength or direction of adaptation has no dependence on the male's own fitness. This means that mtIGEs can either increase or decrease the severity of mother's curse. Whether mother's curse is worsened, ameliorated or unaffected by mtIGEs depends on three factors: mitochondrial relatedness between interacting males and females (r_{mt}), the sign of the effect of the mtIGE on female fitness (B), and

TABLE 4 Posterior estimates of the differences in mean offspring production for each possible pair of female haplotypes in Experiment 2, summed across the four vials and split by "Order of exposure" treatment

Haplotype 1	Haplotype 2	Order of exposure	Difference in offspring production	*SE*	Relative difference
Brownsville	Barcelona	Exposed first	0.35 (−19.34 to 20.24)	10.05	0.07 (0 to 0.2)
Dahomey	Barcelona	Exposed first	−15.13 (−35.67 to 6.02)	10.58	0.17 (0.01 to 0.4)
Dahomey	Brownsville	Exposed first	−15.48 (−36.37 to 5.7)	10.66	0.17 (0.01 to 0.4)
Dahomey	Israel	Exposed first	−17.49 (−38.25 to 3.67)	10.61	0.19 (0.01 to 0.42)
Dahomey	Sweden	Exposed first	−15.78 (−36.36 to 5.19)	10.56	0.17 (0.01 to 0.41)
Israel	Barcelona	Exposed first	2.36 (−17.14 to 22.02)	9.95	0.07 (0 to 0.19)
Israel	Brownsville	Exposed first	2.01 (−17.98 to 21.85)	10.10	0.07 (0 to 0.19)
Sweden	Barcelona	Exposed first	0.65 (−18.97 to 20.38)	9.98	0.07 (0 to 0.2)
Sweden	Brownsville	Exposed first	0.3 (−19.54 to 20.09)	10.08	0.07 (0 to 0.2)
Sweden	Israel	Exposed first	−1.71 (−21.3 to 17.93)	9.97	0.07 (0 to 0.2)
Brownsville	Barcelona	Exposed second	23.52 (5.49 to 42.96)	9.51	0.22 (0.06 to 0.36)*
Dahomey	Barcelona	Exposed second	11.36 (−4.66 to 27.58)	8.17	0.12 (0.01 to 0.27)
Dahomey	Brownsville	Exposed second	−12.16 (−31.36 to 5.94)	9.47	0.14 (0.01 to 0.36)
Dahomey	Israel	Exposed second	−16.78 (−35.87 to 1.04)	9.35	0.19 (0.02 to 0.41)
Dahomey	Sweden	Exposed second	−21.41 (−40.68 to −3.46)	9.47	0.23 (0.04 to 0.46) *
Israel	Barcelona	Exposed second	28.13 (10.47 to 47.4)	9.37	0.25 (0.1 to 0.38) *
Israel	Brownsville	Exposed second	4.62 (−15.59 to 24.87)	10.24	0.08 (0 to 0.21)
Sweden	Barcelona	Exposed second	32.77 (14.76 to 52.33)	9.56	0.28 (0.14 to 0.4) *
Sweden	Brownsville	Exposed second	9.25 (−11.15 to 29.89)	10.40	0.1 (0 to 0.24)
Sweden	Israel	Exposed second	4.64 (−15.37 to 24.75)	10.21	0.08 (0 to 0.2)

Note: Females carrying the Barcelona haplotype tended to have lower offspring production, but only in the "Exposed second" treatment. There was also a difference in offspring production between females carrying the Dahomey and Sweden haplotypes. Asterisks mark statistically significant differences. The numbers in parentheses are 95% credible intervals. The "Relative difference" column gives the absolute difference in means divided by the mean for haplotype 1.

the covariance between the mtIGE and male fitness (absent from the equation). For example, when males tend to interact with positively mitochondrially related females (e.g., sisters), and these females benefit from the males being viable and fertile, mtDNA mutations that enhance male survival/fertility should be selected for, which incidentally lowers mother's curse (Cosmides & Tooby, 1981; Hedrick, 2012; Unckless & Herren, 2009; Wade & Brandvain, 2009; Zhang et al., 2012). The reversal of mother's curse might be especially strong in species in which almost all individuals breed with their siblings, such as fig wasps, because a new male-sterilizing or male-killing mtDNA mutation would rapidly go extinct. However, if instead females benefit when interacting males carry a male-harming mtDNA mutation (e.g., because the mutation kills males and thereby lowers competition on the females), then the mtIGE would cause males to evolve reduced survival. Similarly, if males preferentially interact with mitochondrial nonrelatives ($r_{mt} < 0$), mother's curse will be lessened if males harbouring male-benign (or beneficial) mitochondria are more harmful to females, or worsened when males with male-benign (or beneficial) mitochondria are less harmful to females. Interestingly, these predictions mean that the evolution of inbreeding avoidance via changes to the nuclear genome may create selection for male-sterilizing mitochondrial alleles, whereas the evolution of inbreeding would select against male-sterilizing mtDNA. Thus, mother's curse (and its mitigation or strengthening via mtIGEs) may be an overlooked factor in the evolution of mating systems and sociality.

To sum up, we argue that there are at least three evolutionary mechanisms that can create male-harming mitochondria: ineffective selection on male-harming mutations due to female-limited inheritance ("weak form of Mother's curse"; Dowling & Adrian, 2019; Frank & Hurst, 1996; Havird et al., 2019), selection for male-detrimental mtDNA with pleiotropic benefits to female fitness ("strong form of Mother's Curse"; Camus & Dowling, 2018; Dowling & Adrian, 2019; Havird et al., 2019) and kin selection for male-harming mutations that pleiotropically cause a selectively favourable mtIGE (Cosmides & Tooby, 1981; Engelstädter & Charlat, 2006; Hedrick, 2012; Unckless & Herren, 2009; Wade & Brandvain, 2009; Zhang et al., 2012). These mtIGEs can also lessen mother's curse if they happen to have pleiotropic benefits to males, which might occur when females benefit from their male relatives being healthy and/or fertile. Future work would benefit by focusing on natural populations, for example by testing whether mtIGEs also occur under field conditions, and/or whether interacting males and females show nonzero mitochondrial relatedness at a biologically relevant scale.

Future laboratory-based studies could experimentally create groups of males and females with positive, random or negative mitochondrial relatedness (while standardizing other factors), and then examine the ensuing evolution of mtDNA haplotypes with pronounced male-specific effects.

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AUTHOR CONTRIBUTIONS

TAK and HWSW performed experiments; all authors contributed to the design, analysis and writing.

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DATA AVAILABILITY STATEMENT

A website presenting all of the R code used to analyse and plot the data, along with the raw data files, can be found at https://tomkeaney.github.io/maleMitochondria/. Raw data can also be found at https://doi.org/10.5061/dryad.612jm63zv.

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SUPPORTING INFORMATION

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

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